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MGIGRSEGGRRGALGVLLALGAALLAVGSASEYDYVSFQS
DIGPYQSGRFYTKPPQCVDIPADLRLCHNVGYKKMVLPNL
LEHETMAEVKQQASSWVPLLNKNCHAGTQVFLCSLFAPVC
LDRPIYPCRWLCEAVRDSCEPVMQFFGFYWPEMLKCDKFP
EGDVCIAMTPPNATEASKPQGTTVCPPCDNELKSEAIIEH
LCASEFALRMKIKEVKKENGDKKIVPKKKKPLKLGPIKKK
DLKKLVLYLKNGADCPCHQLDNLSHHFLIMGRKVKSQYLL
TAIHKWDKKNKEFKNFMKKMKNHECPTFQSVFK

(57) Abstract

The invention provides a novel, secreted protein that contains a region homologous to ligand biding domain of a cytokine receptor. This protein, called Frizzled–related protein (FRP), antagonizes the signaling of the Wnt family of cytokines. Extracellular signaling molecules such as the Wnt family members have essential roles as inducers of cellular proliferation, migration, differentiation, and tissue morphogenesis. As Wnt molecules are known to participate in the aberrant growth associated with neoplasia, Wnt antagonists such as FRP are valuable tools which both for understanding oncogenesis and for the design of new cancer therapies.

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HUMAN FRP AND FRAGMENTS THEREOF INCLUDING METHODS FOR USING THEM

Cross-Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 60/050,417, entitled "HUMAN FRP AND FRAGMENTS THEREOF INCLUDING METHODS FOR USING THEM," filed on May 29, 1997, by Rubin et al., and U.S. Provisional Application No. 60/050,495 entitled "HUMAN FRP AND FRAGMENTS THEREOF INCLUDING METHODS FOR USING THEM," filed on June 23, 1997, by Rubin et al., which are incorporated by reference herein.

Background of the Invention

1. Field of the Invention.

This invention is in the field of molecular biology and in particular relates to the identification of a novel human Frizzled Related Protein (FRP) involved in cell growth and differentiation.

2. Description of Related Art.

Extracellular signaling molecules have essential roles as inducers of cellular proliferation, migration, differentiation, and tissue morphogenesis during normal development. These molecules also participate in many of the aberrant growth regulatory pathways associated with neoplasia. In addition, these molecules function as regulators of apoptosis, the programmed cell death that plays a significant role in normal development and functioning of multicellular organisms, and when disregulated, is involved in the pathogenesis of numerous diseases. See e.g. Thompson, C.B., Science 267, 1456–1462 (1995).

Apoptosis is a result of an active cell response to physiological or damaging agents and numerous gene products are involved in signal transduction, triggering and executive steps of the apoptotic pathways. Other proteins do not take part in the apoptotic cascade by themselves but modify cell sensitivity to proapoptotic stimuli. While many genes and gene families that participate in different stages of apoptosis have recently been identified and cloned, because the apoptotic pathways have not been clearly delineated, many novel genes which are involved in these processes await discovery.

The identification and characterization of molecules involved in growth and differentiation is an important step in both the identification of mechanisms of cellular development and oncogenesis and the subsequent conception of novel

therapies based on this knowledge. One group of molecules known to play a significant role in regulating cellular development is the Wnt family of glycoproteins. In vertebrates, this family consists of more than a dozen structurally related molecules, containing 350–380 amino acid residues of which >100 are conserved, including 23–24 cysteine residues. See e.g. Parr, B.A. & McMahon, A. P. (1994) *Curr Opin Genet Dev* 4, 523–8.

Wnt–1, the first Wnt–encoding gene to be isolated, was identified as an oncogene expressed as a result of insertional activation by the mouse mammary tumor virus (Nusse, R., et al., *Nature* 307, 131–6 1984). Subsequently, transgenic expression of *Wnt–1* confirmed that constitutive expression of this gene caused mammary hyperplasia and adenocarcinoma (Tsukamoto et. al., Cell 55, 619–25 (1988)). Targeted disruption of the *Wnt–1* gene revealed an essential role in development, as mouse embryos had severe defects in their midbrain and cerebellum. Thomas et. al., *Cell* 67, 969–76 (1991). In addition, *Wingless* (Wg), the *Drosophila* homolog of *Wnt–1*, was independently identified as a segment polarity gene (Rijsewijk et al., Cell 50, 649–57 (1987)). Gene targeting of other *Wnt* genes demonstrated additional important roles for these molecules in kidney tubulogenesis and limb bud development. See e.g. Parr et al., *Nature* 374, 350–3 (1995); Stark K et al. Nature 372: 679–683, 1994.

Several aspects of Wnt signaling have been illuminated by studies in flies, worms, frogs and mice (Perrimon, N. (1996) *Cell* 86, 513–6; Miller, J. R. & Moon, R. T. (1996) *Genes Dev* 10, 2527–39), but until recently little was known about key events which occur at the external cell surface. Identification of Wnt receptors was hampered by the relative insolubility of the Wnt proteins, which tend to remain tightly bound to cells or extracellular matrix. However, several observations now indicate that members of the Frizzled (FZ) family of molecules including Frzb can function as receptors for Wnt proteins or as components of a Wnt receptor complex. See e.g. He et. al., *Science* 275, 1652–1654 (1997).

The prototype for this family of receptor molecules, *Drosophila frizzled (Dfz)*, was first identified as a tissue polarity gene that governs orientation of epidermal bristles. Vinson et al., *Nature* 329, 549–51 (1987). Cells programmed to express a second *Drosophila Fz* gene, *Fz2*, bind Wg and transduce a Wg signal to downstream components of the signaling pathway. Bhanot et al., Nature 382, 225–30 (1996). Each member of the *Fz* receptor gene family encodes an integral membrane protein with a large extracellular portion, seven putative transmembrane domains, and a cytoplasmic tail. See e.g. Wang et al., *J Biol Chem* 271, 4468–76 (1997). Near the NH2–terminus of the extracellular portion is a cysteine–rich domain (CRD) that is

well conserved among other members of the FZ family. The CRD, comprised of ~110 amino acid residues, including 10 invariant cysteines, is the putative binding site for Wnt ligands. Bhanot et al., Nature 382, 225–30 (1996).

In organisms including frogs, fruit flies, and mice, proteins including Wingless, Armadillo, and Frizzled form part of a signaling cascade that controls crucial events during early embryonic development—particularly gastrulation, the process by which a hollow ball of embryonic cells collapses in on itself, forming the major embryonic tissues. In vertebrates, the signaling pathway—headed by the Wnt family of growth factors contribute to the formation of body axis and the proper development of the central nervous system, kidneys, and limbs. When it is activated inappropriately in adult cells, the pathway can precipitate the formation of tumors. During gastrulation, Fz family members may interact with Wnt to control the proper development of the nervous system and muscles. The coupling of Wnt and Frizzled activates a pathway that leads to the expression of a set of Wnt–responsive genes, including those that encode the transcription factors such as Engrailed and Siamois.

When Wnt mRNA is injected into *Xenopus* embryos in the 4–8 cell stage, the tadpoles develop a second body axis: They can duplicate all or part of the nervous systems from head to tail, and many of their organs are duplicated. Interestingly, during gastrulation, a Wnt family member known as Xwnt–8 serves to "ventralize" the embryo—steering cells in the mesoderm toward forming muscle. Injecting Frzb mRNA into a developing *Xenopus* embryo prior to gastrulation inhibits muscle formation, generating tadpoles that are stunted in appearance, with shortened trunks due to the lack of muscle tissue. The embryos also have enlarged heads, because an abnormal number of mesodermal cells adopt a dorsal fate. Knockout mice have already helped researchers understand a few of the various roles that Wnts play in development. To date, scientists have identified 16 different Wnts that function in vertebrate development. Many Wnts appear to be involved in directing the development of the central nervous system (CNS). Others control the formation of nephrons in the kidney and the proper development of the limbs.

The existence of molecules that have a FZ CRD but lack the seven transmembrane motif and cytoplasmic tail suggests that there is a subfamily of proteins that function as regulators of Wnt activity. Little is known about the activity of SDF5, which was cloned using the signal sequence trap method. FRZB is a heparin–binding molecule thought to be involved in skeletal morphogenesis. Recently Rattner *et al.* cloned cDNAs encoding the murine homologs of Fz family members, and showed that, when artificially linked to the plasma membrane via a

glycolipid anchor, SDF5 and FRZB conferred cellular binding to Wg. Rattner et al., P.N.A.S. 94, 2859-2863 (1997).

The disregulation of Wnt pathways appears to be a factor in aberrant growth and development. Mutations in β-catenin, a protein that accumulates when the Wnt pathway is activated, are associated with tumor development in human colon cancers and melanomas. β-catenins couple with other cellular transcription factors and help to activate Wnt-responsive genes. These results confirm that the Wnt-signaling pathway can play an important role in the embryo and the adult. Ultimately, Wnt transmits its signal by allowing β-catenin to accumulate in the cell cytoplasm. There, β-catenin binds to members of the Tcf-Lef transcription factor family and translocates to the nucleus. When Wnt is absent, β-catenin instead forms a complex with glycogen synthase kinase-3 (GSK-3) and the adenomatous polyposis coli (APC) tumor-suppressor protein. This interaction is associated with the phosphorylation of β-catenin, marking it for ubiquitination and degradation. Wnt permits the accumulation of β-catenin by inhibiting the function of GSK-3. The mutations that drive tumor formation follow a similar strategy. Mutations in APC render the tumor-suppressor protein unable to bind to β-catenin, which remains unphosphorylated and accumulates in the cell, turning on Wnt- responsive genes.

Given the potential complexity of interactions between the multiple members of Wnt and FZ families, additional mechanisms might exist to modulate Wnt signaling during specific periods of development or in certain tissues. What is needed in the art is the identification and characterization of novel effectors of the processes which are related to cellular growth and development. The identification of such mechanisms and in particular, the effectors of these mechanisms is important for understanding and modulating the processes of cellular regulation.

Summary of the Invention

The invention includes nucleotide sequences that encode a novel polypeptide, designated in the present application as "FRP" (Frizzled Related Protein), which is a secreted antagonist of the Wnt signaling pathway and exhibits a number of characteristics which make it a useful tool for studying cell growth and differentiation as well as oncogenesis. As such, this novel protein has a variety of applications in the identification, characterization and regulation of activities associated with cellular function as well as processes associated with oncogenesis.

The invention provides "FRP" (Frizzled Related Protein) polypeptides and fragments thereof and polynucleotide sequences encoding FRP polypeptides. The invention further provides antibodies which are specific for FRP polypeptides and

animals having FRP transgenes. Moreover, the invention provides methods of producing FRP polypeptides and polynucleotide sequences. In addition, the invention provides methods of assaying for FRP in a sample as well as methods for detecting FRP binding partners.

In one embodiment, the invention provides isolated nucleic acid molecules which encode FRP polypeptides. For example, the isolated nucleic acid can include DNA encoding FRP polypeptide having amino acid residues 1 to 313 of Figure 1, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In another embodiment, the invention provides a vector comprising a gene encoding a FRP polypeptide. A host cell comprising such a vector is also provided. By way of example, the host cells may be *E. coli*, yeast or mammalian cells. A process for producing FRP polypeptides is further provided and comprises culturing host cells under conditions suitable for expression of FRP. If desired, the FRP may be recovered.

In another embodiment, the invention provides isolated FRP polypeptide. In one embodiment, FRP of the invention comprises 313 amino acids and includes a signal sequence, Wnt binding domain, a hyaluronic acid binding domain and potential asparagine-linked glycosylation sites. In particular, the invention provides isolated native sequence FRP polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 313 of Figure 1. In a related embodiment, the invention provides chimeric molecules comprising FRP polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule is a factor which includes a FRP fused to a polyhistidine polypeptide sequence. In another embodiment, the invention provides a non-human transgenic animal whose somatic and germ cells contain a transgene comprising human FRP. In yet another embodiment, the invention provides a polypeptide capable of specifically binding a FRP polypeptide such as an antibody specific for a FRP polypeptide. Optionally, the antibody is a monoclonal antibody.

Also included in the invention is a method for regulating cell signaling pathways by inhibiting the interaction of Wnt with Fz receptors by blocking this interaction with FRP molecules. The invention also provides methods for determining the presence of FRP molecules in a sample. The invention also provides a method for determining the presence of Wnt molecules in a sample by screening the sample with FRP. In addition, the invention provides a method for monitoring the course of a neoplastic condition by quantitatively determining the presence of Wnt molecules in a sample by screening the sample with FRP.

In other embodiments, the invention provides methods for using FRP polypeptides and nucleic acids for studying and modulating mechanisms involved in cellular proliferation. In one embodiment, the invention provides a method of modulating cellular phenotype by controlling the level of FRP expression within the cell. In a more specific embodiment, the invention provides a method of inhibiting cellular proliferation and/or differentiation by exposing a cell to FRP.

Brief description of the drawings

Figure 1A shows an SDS/PAGE analysis of heparin-Sepharose purified FRP.

Figure 1B shows a restriction endonuclease map with representations of the human FRP cDNA clones and the coding region of the gene.

Figure 1C shows the predicted FRP amino acid sequence (standard single-letter code).

Figure 2 shows a comparison of the CRDs of FRP and other members of the FZ family.

Figure 3 shows a northern blot analysis showing FRP mRNA expression in normal human adult and embryonic tissues, and in cultured cells.

Figure 4 shows the chromosomal localization of the FRP gene by fluorescent *in situ* hybridization.

Figure 5 shows a southern blot analysis of FRP genomic sequences in different species.

Figure 6 shows the biosynthesis of FRP in M426 cells via a pulse-chase experiment performed with metabolically labeled cells incubated either in the absence or presence of heparin.

Figure 7 shows the dorsal axis duplication in *Xenopus* embryos in response to varying combinations of Wnt and FRP transcripts.

Figures 8A, 8B and **8C** show nucleic acid sequences which encode FRP.

Figure 9 shows the binding of FRP to biotinylated hyaluronic acid in a transblot assay under either nonreducing (−) or reducing (=) conditions.

Figure 10 shows the competition of BHA binding to FRP by various proteoglycans, C.S. is chondroitin sulfate, H.A. is hyaluronic acid, H.A. oligo is hyaluronic acid oligosaccaride. The Ab control consists of a western blot of FRP with rabbit polyclonal antiserum raised against FRP synthetic peptide.

Figure 11 shows the nucleic acid sequence of a FRP 5' flanking genomic sequences.

Figure 12A shows the production of recombinant FRP.

Figure 12B shows immunoblotting of recombinant FRP with peptide antiserum.

Figure 12C shows a polyacrrylamide gel of silver stained recombinant FRP.

Figure 13A shows the interaction between recombinant FRP and Wg in an ELISA format.

Figure 13B shows the immunoblotting of Wg.

Figure 14 shows an ELISA type competition assay showing the ability of soluble FRP to block Wg binding to FRP coated cells.

Figure 15 shows the effects of varying the concentration of heparin in crosslinking reactions between ^{125}I−FRP and Wg, with the crosslinked molecules being immunoprecipitated with an anti−Wg monoclonal antibody and separated by gel electrophoresis.

Figure 16 shows the effects of varying the concentration of unlabelled FRP or FRP derivatives in crosslinking reactions between ^{125}I−FRP and Wg, with the crosslinked molecules being immunoprecipitated with an anti−Wg monoclonal antibody and separated by gel electrophoresis.

Detailed Description of the Invention

Definitions

As used in this application, the following words or phrases have the meanings specified.

The terms "FRP polypeptide" and "FRP" when used herein encompass native sequence FRP and FRP variants (which are further defined herein). The FRP may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

The FRP polypeptide, which may be a fragment of a native sequence, contains a Wnt binding domain. Typically, the FRP polypeptide also includes a hyaluronic acid binding domain.

A "native sequence FRP" is a polypeptide having the same amino acid sequence as an FRP derived from nature. Such native sequence FRP can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence FRP" specifically encompasses naturally–occurring variant forms (*e.g.*, alternatively spliced forms) and naturally–occurring allelic variants of the FRP. In one embodiment of the invention, the native sequence FRP is a mature or full–length native sequence FRP polypeptide comprising amino acids 1 to 313 of Figure 1.

"FRP variant" means a functionally active FRP as defined below having at least about 80% amino acid sequence identity with FRP, such as the FRP polypeptide having the deduced amino acid sequence shown in Figure 1 for a full–length native sequence FRP. Such FRP variants include, for instance, FRP polypeptides wherein one or more amino acid residues are added, or deleted, at the N– or C–terminus of the sequence of Figures 1. Ordinarily, a FRP variant will have at least about 80% or 85% amino acid sequence identity with native FRP sequences, more preferably at least about 90% amino acid sequence identity. Most preferably a FRP variant will have at least about 95% amino acid sequence identity with native FRP sequence of Figures 1.

"Percent (%) amino acid sequence identity" with respect to the FRP sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the FRP sequence, after aligning the sequences in the same reading frame and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer

software such as BLAST software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Percent (%) nucleic acid sequence identity" with respect to the FRP sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the FRP sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising FRP, or a functional fragment thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, or which can be identified by some other agent, yet is short enough such that it does not interfere with activity of the FRP. The tag polypeptide preferably also is sufficiently unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a contaminating component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified to a degree sufficient to obtain N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the FRP natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step (referred to herein as an "isolated and purified polypeptide").

An "isolated" FRP nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with

which it is ordinarily associated in the natural source of the FRP nucleic acid. An isolated FRP nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated FRP nucleic acid molecules therefore are distinguished from the FRP nucleic acid molecule as it exists in natural cells. However, an isolated FRP nucleic acid molecule includes FRP nucleic acid molecules contained in cells that ordinarily express FRP where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking may be accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers may be used in accordance with conventional practice.

"Polynucleotide" and "nucleic acid" refer to single or double–stranded molecules which may be DNA, comprised of the nucleotide bases A, T, C and G, or RNA, comprised of the bases A, U (substitutes for T) , C, and G . The polynucleotide may represent a coding strand or its complement. Polynucleotide molecules may be identical in sequence to the sequence which is naturally occurring or may include alternative codons which encode the same amino acid as that which is found in the naturally occurring sequence (See, Lewin "Genes V" Oxford University Press Chapter 7, pp. 171–174 (1994)). Furthermore, polynucleotide molecules may include codons which represent conservative substitutions of amino acids as described. The polynucleotide may represent genomic DNA or cDNA.

"Polypeptide" refers to a molecule comprised of amino acids which correspond to those encoded by a polynucleotide sequence which is naturally occurring. The polypeptide may include conservative substitutions where the naturally occurring

amino acid is replaced by one having similar properties, where such conservative substitutions do not alter the function of the polypeptide (See, Lewin "Genes V" Oxford University Press Chapter 1, pp.: 9–13 (1994)).

The term "antibody" is used in the broadest sense and specifically covers single anti–FRP monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti–FRP antibody compositions with polyepitopic specificity as well as other recombinant molecules derived from these antibodies. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally–occurring mutations that may be present in minor amounts.

As used herein, "non–FRP binding molecule" is defined as a molecule which does not bind FRP.

As used herein a "binding domain" means that portion or portions of a molecule which confer the ability to bind its target.

As used herein "blocking" means to interfere with the binding of one molecule to another.

As used herein a "sample" means any sample which may contain molecule of interest and includes but is not limited to (1) biological fluids such as solutions comprising blood, lymph, saliva and/or urine and (2) tissues derived from brain, lung, muscle and/or bone.

In order that the invention herein described may be more fully understood, the following description is set forth.

Identification of a Novel Wnt Binding Ligand.

Disclosed herein is a novel human gene product which resembles FZ proteins in that it possesses a conserved FZ CRD, a putative binding domain for Wnt ligands. In contrast to the original members of the FZ family, FRP lacks any transmembrane region or cytoplasmic domain required to transduce Wnt signaling inside the cell. Because it is preferentially distributed to the cell surface or matrix, it is well–positioned to interact with Wnt proteins. Findings disclosed herein indicate that in *Xenopus* embryos FRP inhibits Wnt–dependent axial duplication when various Wnts and FRP are co–expressed. FRP behaves like a dominant–negative receptor in this model system, similar to the effect of the secreted NH2–terminal ectodomain of human FZ5 on axis duplication by XWnt–5A and hFZ5 (He et al. Science 275, 1652–1654 (1997)).

The existence of other molecules besides FRP that have a FZ CRD but lack the seven transmembrane motif and cytoplasmic tail suggests that there is a subfamily of proteins that function as regulators of Wnt activity. Little is known about the activity of SDF5, which was cloned using the signal sequence trap method (Shirozu et al., (1996) Genomics 37, 273–280). FRZB is a heparin–binding molecule thought to be involved in skeletal morphogenesis (Hoang, B., Moos, M., Jr., Vukicevic, S. & Luyten, F. P. (1996) J Biol Chem 271, 26131–7). Recently Rattner *et al.* cloned cDNAs encoding the murine homologs of SDF5, FRZB and FRP, and showed that, when artificially linked to the plasma membrane via a glycolipid anchor, SDF5 and FRZB conferred cellular binding to Wg (Rattner, A., Hsieh, J. C., Smallwood, P. M., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. & Nathans, J. (1997) Proc Natl Acad Sci U S A 94, 2859–2863). Thus, it now appears likely that these molecules can interact with Wnt proteins and modulate their activity.

<u>Compositions of the Invention.</u>

This invention provides the isolation of human FRP which includes a Wnt binding site (also referred to herein as the cysteine rich domain (CRD), the binding site for Wnt ligands, and the FZ CRD motif) and a hyaluronic acid binding sequence (also referred to herein as the hyaluronic acid binding site and the hyaluronic acid binding domain). FRP is a secreted antagonist to Wnt signaling. In addition, the invention provides FRP polypeptide products of the FRP gene. The invention also provides methods for using the expressed FRP to regulate Wnt signaling, and as a detection means for Wnt proteins and associated processes.

The present invention provides the first human protein product of the FRP gene. In one embodiment, human FRP of the invention includes 313 amino acids. Further it includes a signal sequence, a Wnt binding domain, a hyaluronic acid binding sequence and potential asparagine–linked glycosylation sites. In a preferred embodiment, a human FRP includes a Wnt binding domain as shown in the large shaded region of Figure 1C and a hyaluronic acid binding sequence as shown in the small shaded region of Figure 1C. The amino acid sequence of this FRP is shown in Figure 1C.

In another embodiment, a FRP can be joined to another molecule. The FRP may be fused a variety of known fusion protein partners that are well known in the art such as maltose binding protein, *LacZ*, thioredoxin or an immunoglobulin constant region (*Current Protocols In Molecular Biology*, Volume 2, Unit 16, Frederick M. Ausubul et al. eds., 1995; Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L., Damle, N., and Ledbetter, L.(1991) *J.Exp. Med.* 174, 561–566). In a preferred embodiment, this fusion partner is a non–FRP binding molecule so as to prevent difficulties

associated with intramolecular interactions. In the alternative, the FRP can be joined to a detectable label such as a radioactive isotope such as I^{125} or P^{32}, an enzyme such as horseradish peroxidase or alkaline phosphatase, a fluorophore such as fluorescein isothiocyanate or a chromophore *(Current Protocols In Molecular Biology*, Volume 2, Units 10,11 and 14, Frederick M. Ausubul et al. eds., 1995; *Molecular Cloning, A Laboratory Manual*, § 12, Tom Maniatis et al. eds., 2d ed. 1989).

The invention also provides peptides and polypeptides having a specific portion of the FRP such as the Wnt binding domain or the hyaluronic acid binding domain (Current Protocols In Molecular Biology, Volume I, Unit 8, Ausubul et al. eds., 1995; Solid Phase Peptide Synthesis, *The Peptides* Volume II, G. Barany et al., 1980). As with FRP and FRP fusion proteins, these polypeptides can be joined to amino acid tags such as Hemaglutinin or polyhistidine sequences (Krebs et al., Protein Exp. Pur. **6(6)**, 780–788 (1995); Canfield, V.A., Norbeck, L., and Leveson, R., (1996) Biochemistry **35(45)**, 14165–14172), larger molecules such as immunoglobulin constant regions, various functional domains from other proteins and known fusion proteins partners *(Current Protocols In Molecular Biology*, Volume 2, Unit 16, Frederick M. Ausubul et al. eds., 1995; Linsley, P.S., Brady, W., Urnes, M., Grosmaire, L., Damle, N., and Ledbetter, L. (1991) *J.Exp. Med.* **174**, 561–566). In the alternative, the polypeptide can be joined to a detectable label such as a radioactive isotope, an enzyme, a fluorophore or a chromophore.

The polypeptides of the invention can combine in a wide variety of known reagents; typically as a composition comprising an FRP or portion thereof, included therein in a pharmaceutically acceptable carrier such as dextran in a suitable buffer (*Current Protocols In Molecular Biology*, Volume 3, Appendix A, Frederick M. Ausubul et al. eds., 1995; *The Pharmacological Basis of Therapeutics,* Alfred G. Gilman et al eds., 8th ed. 1993).

The invention includes single and double stranded nucleic acid molecules having human FRP gene sequences. An illustrative example of such a molecule is shown in Figure 8. Alternatively, the nucleic acid molecule is represented by the restriction endonuclease map shown in Figure 1B. These nucleic acid molecules can be RNA such as mRNA or DNA such as cDNA. In a preferred embodiment, the nucleic acid molecule or a hybrid thereof can be joined to a detectable label or tag such as P^{32}, biotin or digoxigenein, (*Current Protocols In Molecular Biology*, Volume I, Unit 3, Frederick M. Ausubul et al. eds., 1995).

In another embodiment, a nucleic acid molecule can include a specific portion of the FRP molecule such as the untranslated regulatory regions, the Wnt binding domain or the hyaluronic acid binding domain. In one such embodiment, the nucleic

acid molecule is a deletion mutant which encodes a portion of the region coding for the n–terminus or c–terminus of the FRP protein. In an illustrative embodiment of such a deletion mutant, the deleted sequence encodes the putative FRP signal sequence from figure 1C. In another embodiment, the nucleic acid molecule is a deletion mutant in which an internal portion of the FRP coding region has been removed. These FRP molecules can be joined to other nucleic acid molecules such as those encoding fusion protein partners *(Molecular Cloning, A Laboratory Manual*, § 14 and Appendix F, Tom Maniatis et al. eds., 2d ed. 1989). These specific nucleic acid molecules may also be joined to a tag or a detectable label.

Another embodiment provides a complementary nucleic acid probe which specifically hybridizes to FRP nucleic acid sequences *(Molecular Cloning, A Laboratory Manual*, § 7, 9, 14 and Appendix F, Tom Maniatis et al. eds., 2d ed. 1989). In a preferred embodiment, such a probe hybridizes with the Wnt binding domain which encodes amino acids 57–166 as shown in Figure 1C. For example, the isolated nucleic acid can include DNA encoding FRP polypeptide having amino acid residues 57–166 of Figure 1C, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. Those skilled in the art appreciate that the stringency of the conditions are manipulated by altering the ionic strength and/or temperature of the hybridization, with for example, conditions of higher stringency employing hybridization conditions wherein the complexes are washed under conditions of lower ionic strength and higher temperatures. Thus, prehybridization and hybridization conditions of 42°C in 5X SSPE, 0.3% SDS, 200 μg/ml sheared and denatured salmon sperm DNA, and either 50, 35 or 25% formamide illustrate high, medium and low stringencies, respectively. Variations on such condition are well known in the art (see e.g. U.S. Patent Nos. 5,688,663 and 5,429,921).

Another embodiment provides an antisense nucleic acid which specifically hybridizes to FRP mRNA (Chen, Z., Fischer, R., Riggs, C., Rhim, J. and Lautenberger, J. (1997) Cancer Research **57**, 2013–2019; Aviezer D., Iozzo, R.V., Noonan, D., and Yayon, a., (1997), Mol. Cell Biol. **17(4)**, 1938–1946). Antisense technology entails the administration of exogenous oligonucleotides which bind to a target polynucleotide located within the cells. The term "antisense" refers to the fact that such oligonucleotides are complementary to their intracellular targets, e.g., FRP. See for example, Jack Cohen, OLIGODEOXYNUCLEOTIDES, Antisense Inhibitors of Gene Expression, CRC Press, 1989; and Synthesis 1:1–5 (1988). The FRP antisense oligonucleotides of the present invention include derivatives such as S–oligonucleotides (phosphorothioate derivatives or S–oligos, see, Jack Cohen, supra)

which exhibit enhanced cancer cell growth inhibitory action.

S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligo) in which a nonbridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention may be prepared by treatment of the corresponding O-oligos with 3H-1,2-benzodithiol-3-one-1,1-dioxide which is a sulfur transfer reagent. See Iyer, R. P. et al, J. Org. Chem. 55:4693-4698 (1990); and Iyer, R. P. et al., J. Am. Chem. Soc. 112:1253-1254 (1990), the disclosures of which are fully incorporated by reference herein.

The FRP antisense oligonucleotides of the present invention may be RNA or DNA which is complementary to and stably hybridizes with the first 100 N-terminal codons or last 100 C-terminal codons of the FRP genome or the corresponding mRNA. While absolute complementarity (i.e. a complementary interaction between all polynucleotide moieties) of antisense oligonucleotides is not required, high degrees of complementarity are preferred. Use of an oligonucleotide complementary to this region allows for the selective hybridization to FRP mRNA and not to mRNA specifying other regulatory subunits of protein kinase. Preferably, the FRP antisense oligonucleotides of the present invention are a 15 to 30-mer fragment of the antisense DNA molecule having which hybridizes to FRP mRNA. Optionally, FRP antisense oligonucleotide is a 30-mer oligonucleotide which is complementary to a region in the first 10 N-terminal codons and last 10 C-terminal codons of FRP. Alternatively, the antisense molecules are modified to employ ribozymes in the inhibition of FRP expression. L.A. Couture & D. T. Stinchcomb; *Trends Genet* 12: 510-515 (1996).

The invention further provides an expression vector such as bacteriophage lambda *gt*11, plasmid vectors such as pcDNA, CDM8 and PNTK or retroviral vectors such as those of the pBABE series comprising the FRP cDNA or a portion thereof (*Current Protocols In Molecular Biology*, Volume I, Units 5, 9, 12, Frederick M. Ausubul et al. eds., 1995). Additionally, the invention provides a host vector system in which the expression vector is transfected into a compatible host cell including bacterial strains such as the DH5α strain of E. coli, yeast strains such as EGY48, animal cell lines such as CHO cells and human cells such as HELA cells (*Current Protocols In Molecular Biology*, Volume II, Units 13-16, Frederick M. Ausubul et al. eds., 1995; *Molecular Cloning, A Laboratory Manual*, § 16 and 17, Tom Maniatis et al. eds., 2d ed. 1989). Additionally, the invention provides a method of producing a protein comprising growing the transfected host cell, thereby producing the protein that may be recovered and utilized in a wide variety of applications (*Current Protocols In Molecular Biology*, Volume II, Unit 16, Frederick M. Ausubul et al. eds., 1995).

As discussed in Example 7, an illustrative embodiment of a recombinant expression system is the MDCK/FRP recombinant expression system. Optimization of the MDCK/FRP recombinant expression system may be carried out by techniques known in the art. For example, immunological methods may be employed to screen subpopulations of transfectants to measure the amount of FRP released into the medium. By successive screening and subculturing, clonal lines expressing greater amounts of FRP protein may be obtained. In addition, the illustrative regimen for harvesting conditioned medium involves subculturing into a large number of T-175 flasks and cycling monolayers from serum-containing to serum-free medium several times may be streamlined by utilizing alternative devices such as cell factories and/or microcarriers, and then determine whether multiple successive rounds of FRP-rich, serum-free conditioned medium can be collected once confluent monolayers are generated with serum-containing medium. Such measures can reduce the time and cost of producing large quantities of recombinant protein to be used for structural analysis, biochemical and biological studies.

The asymmetry of FRP elution in the heparin-HPLC optical density profile, and the breadth of the FRP-crossreactive bands suggest that current preparations of recombinant protein are heterogeneous (Figure 12). This is also evident in naturally occurring FRP, as amino-terminal sequence analysis revealed two distinct sequences that differed from each other by a three-amino acid stagger. Finch, et al., P.N.A.S. 94: 6770-6775 (1997). To identify the source of this heterogeneity, amino acid sequence analysis of purified recombinant FRP protein can performed to evaluate the purity of the preparation and indicate whether such differences account for at least some of the apparent heterogeneity. Given the presence of two potential asparagine-linked glycosylation sites in the FRP sequence, variation in glycosylation also might contribute to heterogeneity. This possibility can be tested by expressing site-directed mutants lacking the glycosylation sites; the mobility and breadth of immunocrossreactive bands corresponding to these derivatives will indicate whether FRP normally is glycosylated at these sites and whether this is responsible for heterogeneity. The resolution of putative FRP variants by additional chromatography (ion exchange or hydrophobic interaction, for instance) or their elimination by removal of glycosylation sites is also possible using art accepted techniques and will facilitate structural analysis by methods such as X-ray diffraction or NMR that require homogeneous preparations.

The MDCK/FRP expression system can be used to produce FRP derivatives for structural analysis. Besides mutants lacking glycosylation sites, truncated variants may can be expressed to assess the significance of different structural elements.

While the epitopes and regions associated with the function and integrity of the cysteine–rich domain (CRD) are of significant interest, other regions of the molecule presumably responsible for binding to proteoglycan can also be examined. Such structural studies can be used to determine the disulfide–bonding pattern in the CRD, and consequently, to engineer substitutions of paired cysteine residues to determine the significance of individual disulfide bond–dependent peptide loops. Derivatives can be tested in Wnt–binding ELISAs (for example as shown in Figure 13) and in biological assays involving β–catenin stabilization or other manifestations of Wnt activity. Miller et al., Genes Dev. 10: 2527–2539, (1996). The ELISA format disclosed in Example 8 is particularly well suited for a quantitative comparison of the FRP analogs. Because the heparin–binding and immunological properties of the derivatives will vary, one can express them in pcDNA vectors designed to add histidine and Myc tags to the recombinant protein. In this way, it is possible to purify the various derivatives on nickel–affinity resin and visualize them with antibody directed against the Myc or histidine epitopes.

Reports indicate that there are several other secreted Frizzled–related proteins of similar size and perhaps function. (See e.g., Rattner, et al., P.N.A.S. 94:2859–2863 (1997)). The MDCK/pcDNA expression system is well suited for the production of other secreted FRP molecules. In this way one can compare and contrast the binding and biological properties of multiple secreted FRPs to better understand their unique functions.

Notwithstanding the results obtained with FRP expression in MDCK cells, other recombinant systems may prove more useful for certain applications. For example, for NMR solution analysis, proteins must be uniformly labeled with various non–standard isotopes (^{2}H, ^{13}C, ^{15}N). The medium required to achieve this labeling in mammalian cell culture is very expensive. As an alternative, one may express CRD–containing constructs in yeast (*Pichia pasteuris*), bacteria such as E.coli or baculovirus/insect cell expression systems, where isotope–labeling should be more straightforward.

Further, in accordance with the practice of this invention, FRP molecules of the invention can have amino acid substitutions in the amino acid sequence shown in Figure 1C (*Current Protocols In Molecular Biology*, Volume I, Unit 8, Frederick M. Ausubul et al. eds., 1995). The only requirement being that substitutions result in human FRP that retains the ability to bind the Wnt molecule. These amino acid substitutions include, but are not necessarily limited to, amino acid substitutions known in the art as "conservative".

For example, it is a well-established principle of protein chemistry that certain amino acid substitutions, entitled "conservative amino acid substitutions," can frequently be made in a protein without altering either the conformation or the function of the protein. Such changes include substituting any of isoleucine (I), valine (V), and leucine (L) for any other of these hydrophobic amino acids; aspartic acid (D) for glutamic acid (E) and vice versa; glutamine (Q) for asparagine (N) and vice versa; and serine (S) for threonine (T) and vice versa. Other substitutions can also be considered conservative, depending on the environment of the particular amino acid and its role in the three-dimensional structure of the protein. For example, glycine (G) and alanine (A) can frequently be interchangeable, as can alanine and valine (V). Methionine (M), which is relatively hydrophobic, can frequently be interchanged with leucine and isoleucine, and sometimes with valine. Lysine (K) and arginine (R) are frequently interchangeable in locations in which the significant feature of the amino acid residue is its charge and the differing pK's of these two amino acid residues are not significant. Still other changes can be considered "conservative" in particular environments.

FRP derivatives can be made using methods known in the art such as site-directed mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., *Nucl. Acids Res., 13*:4331 (1986); Zoller et al., *Nucl. Acids Res., 10*:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., *Philos. Trans. R. Soc. London SerA*, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the FRP variant DNA. Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, *The Proteins*, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

As discussed above, redundancy in the genetic code permits variation in FRP gene sequences. In particular, one skilled in the art will recognize specific codon preferences by a specific host species and can adapt the disclosed sequence as preferred for a desired host. For example, preferred codon sequences typically have rare codons (i.e., codons having a useage frequency of less than about 20% in known

sequences of the desired host) replaced with higher frequency codons. Codon preferences for a specific organism may be calculated, for example, by utilizing codon usage tables available on the INTERNET at the following address: http://www.dna.affrc.go.jp/~nakamura/codon.html. Nucleotide sequences which have been optimized for a particular host species by replacing any codons having a useage frequency of less than about 20% are referred to herein as "codon optimized sequences."

Additional sequence modifications are known to enhance protein expression in a cellular host. These include elimination of sequences encoding spurious polyadenylation signals, exon/intron splice site signals, transposon-like repeats, and/or other such well-characterized sequences which may be deleterious to gene expression. The GC content of the sequence may be adjusted to levels average for a given cellular host, as calculated by reference to known genes expressed in the host cell. Where possible, the sequence may also be modified to avoid predicted hairpin secondary mRNA structures. Other useful modifications include the addition of a translational initiation consensus sequence at the start of the open reading frame, as described in Kozak, *Mol. Cell Biol.*, 9:5073-5080 (1989). Nucleotide sequences which have been optimized for expression in a given host species by elimination of spurious polyadenylation sequences, elimination of exon/intron splicing signals, elimination of transposon-like repeats and/or optimization of GC content in addition to codon optimization are referred to herein as an "expression enhanced sequence."

The present invention also provides an antibody which specifically recognizes and binds an epitope on a FRP, e.g. the Wnt binding domain or the hyaluronic acid binding domain of an FRP (*Current Protocols In Molecular Biology*, Volume II, Unit 11, Frederick M. Ausubul et al. eds., 1995; Kohler, G., and Milstein, C., Nature, (1975) **256**, 495-497). A rabbit polyclonal antiserum raised against a synthetic peptide corresponding to a portion of the FRP amino-terminal sequence has been used for the detection of FRP by immunoblotting, immunoprecipitation and ELISA. A rabbit polyclonal antiserum raised against full-length recombinant FRP has also been generated. A related embodiment consists of an anti-idiotype antibody which specifically recognizes and binds antibody generated to an FRP epitope. Related embodiments further provide for single chain and humanized forms of these antibodies (United States Patent No. 5,569,825 to Lonberg et al., issued October 29, 1996; Bei, R., Schlom, J., and Kashmiri, S., (1995) J. *Immunol. Methods* **186(2)** 245-255; Park, S., Ryu, C.J., Gripon, P., Guguen-Guillouzo, C., and Hong, H.J. (1996) *Hybridoma* **15(6)** 435-441). These antibodies may be linked to a detectable label such as one selected from the group consisting of radioactive isotopes, enzymes,

fluorophores or chromophores (*Current Protocols In Molecular Biology*, Volume II, Units 11, 14, Frederick M. Ausubul et al. eds., 1995).

Methods of the Invention.

The invention further provides methods of modulating cellular development. In one embodiment, the method includes the steps of contacting a Wnt molecule with FRP or a portion of the FRP molecule. This contact blocks an interaction between the Wnt molecule and a Fz receptor, thereby inhibiting a cellular process such as proliferation and/or differentiation and/or migration (Tan, P., Anasetti, C., Hansen, J., Melrose, J., Brunvand, M., Bradshaw, J., Ledbetter, J. and Linsley, P., (1993) J. Exp. Med. **177**, 165–173). In a preferred embodiment of this method, the cell is a tumor cell (Estrov, Z. Kurzrock, R., Estey, E. Wetzler, M., Ferrajoli, A., Harris, D., Blake, M., Gutterman, J.U. and Talpaz, M. (1992) *Blood* **79(8)** 1938–1945).

The invention further provides a method for blocking Wnt and Fz receptor binding. This method involves administering FRP to a subject. The FRP so administered must be in an amount sufficient to block the binding between a Wnt molecule and a Fz receptor.

The present invention also provides a method for modulating cellular differentiation in a subject. One such method includes administering cells transfected with an FRP gene (*Current Protocols In Molecular Biology*, Volume I, Unit 9, Frederick M. Ausubul et al. eds., 1995). Once administered, these transfected cells express recombinant FRP in an amount sufficient to block the interactions between a Wnt molecule and Wnt receptor, thereby inhibiting cell proliferation and/or differentiation. In these methods, the FRP gene can be manipulated in one of a number of ways as is well known in the art such as through the use of a plasmid or viral vectors *(Molecular Cloning, A Laboratory Manual*, § 1 and Appendix F, Tom Maniatis et al. eds., 2d ed. 1989). The FRP gene can be inserted into donor cells by a nonviral physical transfection of DNA, by microinjection of RNA or DNA, by electroporation, via chemically mediated transfection or by one of a variety of related methods of manipulation *(Molecular Cloning, A Laboratory Manual*, § 16, Tom Maniatis et al. eds., 2d ed. 1989).

Using the molecules disclosed herein, the invention provides methods to investigate the impact of FRP on effectors of Wnt signaling. In particular, one can measure the steady–state level of soluble β–catenin in cells exposed to Wnt stimulation in the presence or absence of FRP. As in a study showing that *Drosophila* Frizzled 2 (Df2) can mediate Wg–dependent stabilization of armadillo (*Drosophila* beta–catenin homolog), one can treat clone 8 cells expressing endogenous Dfz2

and/or Dfz2–transfected S2 cells with Wg–containing medium preincubated with FRP or vehicle control. Bhanot et al., Nature 382: 225–230, (1996). If FRP functions as a Wg antagonist in this cellular system, consistent with its effect in the *Xenopus* embryo dorsal axis duplication assay, lower levels of armadillo will be seen when cells are exposed to medium preincubated with FRP. Such a result was recently described in another experimental model: MCF–7 human mammary carcinoma cells transfected with an FRP construct showed a marked decrease in cellular β–catenin relative to vector control transfectants, Melkonyan, et al., P.N.A.S. 94:13636–13641 (1997) (in this article FRP was referred to as SARP2). Thus, one can test the ability of recombinant FRP protein to reduce the β–catenin content of this and other cell lines. If documented, this can serve as a convenient, semi–quantitative, functional assay of FRP derivatives. As additional markers of Wnt signaling are delineated, one can test the effect of FRP on these parameters. This can involve Tcf/LEF–1–beta–catenin–dependent gene expression (see e.g., Molenaar, et al., Cell 86:391–399 (1996), changes in cytoskeleton or cell cycle progression. The intent will be to determine whether all manifestations of Wnt signaling are blocked by FRP, or only certain pathways.

The invention further provides a method for determining the presence of FRP nucleic acid and peptide sequences in a sample. This method includes screening a sample with FRP nucleic acid molecules or antibodies via procedures such as reverse transcriptase Polymerase Chain Reaction, and northern and Southern and western protocols as is well known in the art (*Current Protocols In Molecular Biology*, Volume I and III, Units 16, 2 and 4, Frederick M. Ausubul et al. eds., 1995).

As disclosed in Example 3 below, Northern blot analysis of RNA from adult and embryonic organs indicate that FRP is expressed widely, though not ubiquitously in humans. Subsequent *in situ* hybridization analysis of mouse embryonic tissue confirms this interpretation (see Example 3 below). By surveying the pattern of FRP expression during development and in the adult with a combination of *in situ* hybridization and immunohistochemistry, one can identify a set of contexts in which this gene is active. Additionally, one can extend the analysis to models of wound repair in which processes such as cell proliferation, migration and apoptosis are particularly active.

Similarly, FRP sequences and expression can be examined in tumor specimens. For example tumor samples can be screened for evidence of FRP mutations or hypermethylation of regulatory regions associated with the absence of gene expression (see e.g. *Current Protocols In Molecular Biology*, Volumes I and II, Units 3 and 12, Frederick M. Ausubul et al. eds., 1995).

A number of approaches that are well known in the art can be taken to study the regulation of FRP expression. An illustrative approach involves the use of various cytokines and/or growth conditions to assess the impact of different external agents or environmental factors on expression. Multiple FRP-expressing cells can be included in such analyses, as preliminary observations suggest that cells vary in their response to these kinds of stimuli. Complementary to these experiments is the identification and analysis of the FRP promoter region as shown in Figure 11. Sequencing of the presumptive promoter lying 5' upstream of the coding sequence can be followed by subcloning into reporter constructs and transfection into cell lines for functional analysis. Upon confirmation of promoter activity, one can pinpoint the sequences responsible for regulating expression by a combination of deletional analysis and computer-based searches to locate potential binding sites for transcription factors. If putative binding sites are identified, their relevance can be tested by expression of corresponding reporter constructs, gel shift and supershift experiments, and co-expression of promoter reporter constructs with mRNA promoting the expression of the corresponding transcription factor. Xu, et al., P.N.A.S. 93:834-838 (1996). The functionality of putative promoter sequences with respect to determining the spatiotemporal distribution of FRP expression can also be tested by using reporter constructs in transgenic mice.

The invention further provides a method for determining the presence of a Wnt molecule in a sample. This method includes adding a FRP to the sample. The FRP so added can recognize and bind Wnt molecule that is present in the sample. This binding results in a FRP/Wnt complex that can be detected. The presence of the complex is indicative of the presence of the Wnt molecule in the sample. In a variation of this method, detection includes contacting the complex with an antibody which recognizes and binds the complex (*Current Protocols In Molecular Biology*, Volume II, Unit 11, Frederick M. Ausubul et al. eds., 1995). The antibody/complex so bound can be detected. The antibody can be either monoclonal or polyclonal. Further, the antibody can be bound to a matrix such agarose, sepharose, or a related type of bead (*Current Protocols In Molecular Biology*, Volume II, Unit 11, Frederick M. Ausubul et al. eds., 1995). In addition, the antibody may be labeled with a detectable marker such as a radioactive isotope, an enzyme, a fluorophore or a chromophore.

The preparation of ample quantities of purified FRP protein as disclosed in Example 7 below is useful for studying the presumed interactions of FRP and Wnt polypeptides. With sufficient amounts of FRP, multiple experimental designs can be employed to test the hypothesis that FRP and Wnts engage in a direct interaction. The

affinity and specificity of the suspected binding interactions can be determined, as well as the potential requirement for co–factors such as proteoglycan. Experimental models that rely on constitutive expression of FRP may yield important information about the effects of FRP on cell function. However, use of recombinant protein will enable one skilled in the art to control the timing and amount of FRP exposure, and consequently, assess its effects in a more quantitative manner. Moreover, a satisfactory recombinant expression system will be the cornerstone of detailed structure–function analysis.

As illustrated in Example 8, the invention provides methods to evaluate the interaction between FRP and FRP–binding partners. The results obtained with the FRP–Wg binding ELISA in Example 8 establish that this is a useful model for the study of FRP interactions with Wnt proteins. A variety of modifications of such studies are contemplated such as those that identify cofactors in these interactions. For example, the Wg content of purified and crude preparations can be normalized by immunoblot analysis, and the FRP–binding of both Wg samples can be compared in the ELISA disclosed in Example 8. Because the conditions used to purify Wg are relatively gentle, any appreciable decrease in binding activity of the enriched Wg preparation could be attributable to loss of a cryptic co–factor rather than denaturation of the Wg protein. If such a loss were observed, a leading candidate for the putative co–factor would be soluble glycosaminoglycan. To examine this, one can treat the crude material with heparitinases, or add exogenous proteoglycan to purified Wg in the ELISA and observing whether binding activity is restored.

Utilizing purified Wg one can estimate the affinity of the apparent FRP–Wg interaction. Either by using purified Wg directly in the FRP–binding ELISA, or simply to quantify the amounts of Wg in the starting material and bound in the ELISA microtiter well, it is possible to determine the concentration of bound versus free Wg in the assay and perform a Scatchard analysis to provide a useful indication of the affinity characterizing FRP–Wnt interactions.

As mentioned above, the ELISA format illustrated in Example 8 can be employed to examine Wnt binding of various FRP analogs. One can investigate the Wg–binding of FRP truncation mutants and site–directed variants lacking the asparagine–linked glycosylation sites. Further, the binding of additional site–directed FRP mutants and other secreted FRPs can be assessed. The substitution other Wnt family members for Wg in the binding assay will provide additional useful information.

Cell–free binding assays that complement the disclosed ELISA include ligand–receptor, cross–linking analysis. While in classical covalent binding studies a

radioisotope–labeled ligand is employed, one can use ^{125}I–FRP as tracer in combination with unlabeled Wg (or other available soluble Wnt protein) and a cross–linking agent such as bis(sulfosuccinimidyl) suberate. Subsequent immunoprecipitation with antibody to Wg (or the appropriate epitope if a tagged Wnt molecule is involved), followed by SDS–PAGE and autoradiography will provide evidence of a direct FRP–Wg interaction.

Cellular binding assays can also be performed, using labeled FRP as tracer. While Wnt protein may be accessible at the cell surface, alternative models such as fusion proteins can be employed to anchor Wnt in the membrane and facilitate detection. Parkin, et al., Genes Dev. 7:2181–2193 (1993). While in such studies, the ligand–receptor relationship of Wg and FRP will be reversed; nonetheless, binding will occur and be suitable for Scatchard analysis. A quantitative measure of this condition can be obtained by assaying Wg–binding of the tracer in the ELISA disclosed in Example 8.

The present invention also provides a method for monitoring the course of a neoplastic condition in a subject. This method includes quantitatively determining in a first sample, from the subject, the presence of a Wnt molecule by detection method such as those commonly utilized in immunohistochemistry (*Current Protocols In Molecular Biology*, Volume II, Unit 14, Frederick M. Ausubul et al. eds., 1995). The amount so determined is compared with an amount present in a second sample from the subject. Each sample is taken at a different point in time. A difference in the amounts determined is indicative of the course of the neoplastic condition.

As outlined above, FRP plays a role in neoplasia. Ectopic expression of Wnt–1 caused hyperplasia and adenocarcinoma in mouse mammary gland. (See e.g., Tsukamoto, et al., Cell 55:619–625 (1988)). Stabilization of beta–catenin–a hallmark of Wnt signaling – occurs with high frequency either as a consequence of mutation in the beta–catenin or APC genes in human colon cancer and melanoma. (See e.g., Korinek, et al., Science 275:1784–1787 (1997)). The ability of FRP to inhibit Wnt signaling in the *Xenopus* axis duplication assay (Finch, et al., P.N.A.S. 94:6770–6775 (1997) and to decrease intracellular beta–catenin concentration in MCF–7 transfectants (Melkonyan, et al., P.N.A.S. 94:13636–13641 (1997)) suggests that FRP might function to suppress signaling in a pathway that can contribute to malignancy. FRP expression in MCF–7 cells apparently caused an increase in the number of cells undergoing apoptosis (Melkonyan, et al., P.N.A.S. 94:13636–13641 (1997)). Moreover, deletions or loss of heterozygosity have been described at the *FRP* chromosomal locus, 8p11.1–12, in association with kidney, breast, prostate, bladder and pancreas carcinoma and astrocytoma. See e.g., Mitelman, et al., Nature Genet 15:

417-474 (1997). Translocations at this locus were reported to occur in cases of myeloproliferative disease, T-ALL and T-PLL (Mitelman, et al., Nature Genet 15: 417-474 (1997)). Taken together, these observations raise the possibility that FRP may act as a tumor suppressor by inhibiting Wnt signaling and promoting apoptosis; loss of this function foster tumorigenesis.

One can perform a screen of FRP mRNA expression in a large sample of tumor cell lines. This can be accompanied by Southern blotting of restriction digests of genomic DNA from lines lacking FRP expression to look for gross changes in *FRP* gene structure. If no differences are seen relative to a normal control, more sensitive methods of detecting point mutations such as single strand conformation polymorphism (SSCP) can be used. Humphries, et al. Clin. Chem. 43:427-435 (1997). To facilitate this analysis, exon-intron boundaries of the FRP gene can be determined; and with this information, PCR primers can be designed to assist in the investigation of the gene structure of coding sequence. Any evidence of mutation can be confirmed by nucleotide sequence analysis. In addition to studying cell lines, one can screen paired tumor-bearing and tumor-free tissue specimens from individual patients. Gene targeting of FRP can also provide evidence of tumor suppressor function, if it resulted in mice that were prone to malignancy. Animals with this phenotype will serve as a useful model for the investigation of molecular events that culminate in neoplasia.

The invention also provides screening method for molecules that react with a Wnt or FRP molecules by a two-hybrid screen (Janouex-Lerosey I., Jollivet, F., Camonis, J., Marche, P.N., and Goud, B., (1995) J. Biol. Chem. **270(24)**, 14801-14808) or by Western or Far Western techniques (*Current Protocols In Molecular Biology*, Volume I, Unit 11, Frederick M. Ausubul et al. eds., 1995; Takayama, S., and Reed, J.C. (1997) Methods Mol. Biol. **69**, 171-184). One method includes separately contacting each of a plurality of samples. Each sample contains a predefined number of cells. Further, each sample contains a predetermined amount of a different molecule to be tested.

Use of FRP Upstream Control Sequences For Evaluating Regulatory Processes

The genomic FRP control sequences of the present invention, whether positive, negative, or both, may be employed in numerous various combinations and organizations to assess the regulation of FRP. Moreover, in the context of multiple unit embodiments and/or in embodiments which incorporate both positive and negative control units, there is no requirement that such units be arranged in an adjacent head-to-head or head-to-tail construction in that the improved regulation

capability of such multiple units is conferred virtually independent of the location of such multiple sequences with respect to each other. Moreover, there is no requirement that each unit include the same positive or negative element. All that is required is that such sequences be located upstream of and sufficiently proximal to a transcription initiation site.

To evaluate FRP regulatory elements in the context of heterologous genes, one simply obtains the structural gene and locates one or more of such control sequences upstream of a transcription initiation site. Additionally, as is known in the art, it is generally desirable to include TATA–box sequences upstream of and proximal to a transcription initiation site of the heterologous structural gene. Such sequences may be synthesized and inserted in the same manner as the novel control sequences. Alternatively, one may desire to simply employ the TATA sequences normally associated with the heterologous gene. In any event, TATA sequences are most desirably located between about 20 and 30 nucleotides upstream of transcription initiation.

Preferably the heterologous gene is a gene which encodes an enzyme which produces colorimetric or fluorometric change in the host cell which is detectable by in situ analysis and which is a quantitative or semi–quantitative function of transcriptional activation. Exemplary enzymes include esterases, phosphatases, proteases (tissue plasminogen activator or urokinase) and other enzymes capable of being detected by activity which generates a chromophore or fluorophore as will be known to those skilled in the art. A preferred example is E. coli β–galactosidase. This enzyme produces a color change upon cleavage of the indigogenic substrate indolyl–B–D–galactoside by cells bearing β–galactosidase (see, e.g., Goring et al., Science, 235:456–458 (1987) and Price et al., Proc. Natl. Acad. Sci. U.S.A., 84:156–160 (1987)). Thus, this enzyme facilitates automatic plate reader analysis of FRP control sequence mediated expression directly in microtiter wells containing transformants treated with candidate activators. Also, since the endogenous β–galactosidase activity in mammalian cells ordinarily is quite low, the analytic screening system using β–galactosidase is not hampered by host cell background.

Another class of reporter genes which confer detectable characteristics on a host cell are those which encode polypeptides, generally enzymes, which render their transformants resistant against toxins, e.g., the neo gene which protects host cells against toxic levels of the antibiotic G418; a gene encoding dihydrofolate reductase, which confers resistance to methotrexate, or the chloramphenicol acetyltransferase (CAT) gene (Osborne et al., Cell, 42:203–212 (1985)). Genes of this class are not preferred since the phenotype (resistance) does not provide a convenient or rapid

quantitative output. Resistance to antibiotic or toxin requires days of culture to confirm, or complex assay procedures if other than a biological determination is to be made.

Use of FRP Nucleic Acids in the Generation of Transgenic Animals.

Nucleic acids which encode FRP or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding FRP can be used to clone genomic DNA encoding FRP in accordance with established techniques and the genomic or cDNA sequences can then be used to generate transgenic animals that contain cells which express DNA encoding FRP. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for FRP transgene incorporation with inducible and tissue–specific control elements. Illustrative inducible and tissue specific control sequences include the mouse mammary tumor long terminal repeat (MMTV LTR) and the tetracycline elements respectively (see e.g. Hennighausen et al., J Cell Biochem Dec;59(4):463–72 (1995).

Transgenic animals that include a copy of a transgene encoding FRP introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding FRP. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, non–human homologues of FRP can be used to construct a FRP "knock out" animal which has a defective or altered gene encoding FRP as a result of homologous recombination between the endogenous gene encoding FRP and altered genomic DNA encoding FRP introduced into an embryonic cell of the animal. For example, cDNA encoding FRP can be used to clone genomic DNA encoding FRP in accordance with established techniques. A portion of the genomic DNA encoding FRP can be deleted or replaced with another gene, such as a gene encoding a

selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li et al., Cell, 69:915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the FRP polypeptide.

Advantages of the invention.

FRP is a previously undescribed human gene product that is involved in regulating cellular growth and differentiation. This novel polypeptide antagonizes Wnt action. As a secreted antagonist which competes for a factor known to regulate cellular growth and development, FRP is a prototype for molecules that function as endogenous regulators of cytokine activity. As such, this novel protein has a variety of applications in the identification, characterization and regulation of activities associated with the Wnt family of cytokines.

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EXAMPLES

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which the invention pertains. The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

EXAMPLE 1

Purification and Physical Characterization of the FRP Protein.

Conditioned-medium collection, ultrafiltration, heparin-Sepharose affinity chromatography, and SDS/PAGE were performed as described (Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989) Proc Natl Acad Sci U S A 86, 802-6). Hepatocyte growth factor/scatter factor (HGF/SF)-containing fractions were identified by immunoblotting. Occasionally heparin-Sepharose fractions were processed by reverse-phase C_4 HPLC (Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989) Proc Natl Acad Sci U S A 86, 802-6) to enhance purity of FRP. Gels were fixed and silver-stained using the reagents and protocol from Bio-Rad.

During the isolation of HGF/SF from human embryonic lung fibroblast culture fluid, a 36 kDa polypeptide which co-purified with HGF/SF following a variety of chromatography procedures was identified (Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H. Taylor, W. G., Cech, A. C., Hirshfield, D. W., Wong, J., Miki, T., Finch, P. W. & et al. (1991) Proc Natl Acad Sci U S A 88, 415-9). Because the co-migration of this protein and HGF/SF suggested that it might regulate growth factor activity, a preparative scheme was devised to obtain sufficient quantities for study. This was accomplished by conservative pooling of fractions eluting from heparin-Sepharose resin with 1.0 M NaCl, once it became evident that a portion of the 36 kDa protein emerged after the HGF/SF-containing fractions. Protein obtained in this manner was sufficiently pure and abundant for structural and limited functional analysis (Fig. 1A).

<u>Microsequencing.</u>

Approximately 30 µg of protein was loaded onto an Applied Biosystems gas-phase protein sequenator. Forty rounds of Edman degradation were carried out, and phenylthiohydantoin amino acid derivatives were identified with an automated on-line HPLC column (model 120A, Applied Biosystems).

Figure 1 provides illustrations of these protocols and results. Figure 1 consists of a series of panels. Panel (A) shows an SDS/PAGE analysis of heparin-Sepharose purified FRP. Approximately 200 ng of protein was resolved in a 4-20% polyacrylamide minigel (Novex) under reducing (+) or non-reducing (-) conditions, and subsequently stained with silver. The position of molecular mass markers is indicated at the right.

EXAMPLE 2

Molecular Cloning and Characterization of FRP Nucleic Acid Sequences.

Four pools of 26–base degenerate oligonucleotides were synthesized on the basis of either of two segments of amino acid sequence determined by microsequencing of purified FRP. Two pools corresponding to the sequence NVGYKKMVL contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for the first Val and Gly; one subset terminated with bases CT and the other with TT. Two additional pools, corresponding to the sequence FYTKPPQXV, contained all possible codon combinations except for the substitution of inosine residues in the third positions of the codons for both Pro residues; one subset contained four codon options for Ser in the X position, while the other had the remaining two. Oligonucleotide pools were labeled and used to screen an oligo (dT)–primed M426 cDNA library as previously described (Finch, P. W., Rubin, J. S., Miki, T., Ron, D. & Aaronson, S. A. (1989) Science 245, 752 5).

Microsequencing of the purified 36 kDa protein yielded two amino–terminal sequences, one beginning three residues downstream from the other. Positive identifications were made in 37 of the first 40 cycles of Edman degradation, as follows: FQSDIGPYQSGRFYTKPPQXVDIPADLRLXXNVGYKKMVL (X denotes inability to make an amino acid assignment). Degenerate oligonucleotides corresponding either to sequence FYTKPPQXV or NVGYKKMVL were used to probe a M426 cDNA library. An initial screening of 10^6 plaques yielded approximately 350 clones recognized by probes derived from both peptide segments. Restriction digestion of several plaque–purified phage DNAs revealed two classes of inserts. Selected cDNA inserts were analyzed by restriction endonuclease digestion. The nucleotide sequence of the FRP cDNAs was determined by the dideoxy chain–termination method. To search for homology between FRP and any known protein, we analyzed the GenBank, PDB, SwissProt and PIR protein sequence data bases. Alignments were generated with the program PileUp from the Wisconsin Package Version 8 (Genetics Computer Group; Madison WI). Mapping (Fig. 1B) and sequence analysis (Fig. 1C) of a representative from each class, designated HS1 and HS8, demonstrated that they were overlapping cDNAs. HS1 was ~2 kb in length and contained a 942–bp open reading frame; HS8 encoded a portion of the 942–bp open reading frame as well as approximately 0.3 kb of cDNA extending upstream of the ATG start codon. The putative start codon, located at position 303 in the HS1 sequence, was flanked by sequence that closely matched the proposed GCC(G/A)CCATGG consensus sequence for optimal initiation by eukaryotic

ribosomes (Kozak, M. (1987) Nucleic Acids Res 15, 8125–48). An upstream in-frame stop codon was not present.

As expected for a secreted protein, a hydrophobic 26–amino acid segment at the NH2–terminus likely functions as a signal peptide. The experimentally determined protein sequence begins 11 residues downstream from the presumptive signal sequence, suggesting additional processing or incidental proteolysis. There was complete agreement between the predicted and observed amino acid sequences; the three undefined residues in the latter corresponded to Cys57, Cys67 and His68, residues which typically are undetectable or have low yields following Edman degradation. Two overlapping sequences in the COOH terminal region fulfill the criteria for a consensus binding site to a hyaluronic acid (Yang, B., Yang, B. L., Savani, R. C. & Turley, E. A. (1994) Embo J 13, 286–96) (Fig. 1C). Two potential asparagine–linked glycosylation sites are also present. A consensus polyadenylation signal was not identified in the cDNA sequence, raising the possibility that the cDNA clones from this oligo–dT primed library resulted from internal priming at an adenine–rich region.

Once a FRP cDNA was isolated, FRP genomic sequences were readily identified by methods that are well known in the art. Briefly, a human genomic DNA library (Stratagene) was screened by Southern blotting with two different human FRP cDNA probes: pF2 insert was used to identify genomic fragments containing any of the coding sequence; SalI–BstXI fragment (bp 417–781, according to numbering scheme for pF2) was used to identify genomic fragment(s) containing sequence encoding the cysteine–rich domain (CRD). Phage containing DNA that hybridized with FRP probes were plaque–purified, and portions of the genomic DNA inserts were then isolated and sequenced. A portion of the genomic sequence including the 5' flanking region is illustrated in Figure 11.

Relationship to the FZ Protein Family.

Search of several protein databases revealed significant homology of a portion of the predicted amino acid sequence to a specific region conserved among members of the FZ family (Fig. 2). The observed homology is confined to the extracellular CRD of FZ, a region consisting of ~110 amino acid residues that includes 10 cysteines and a small number of other invariant residues. This domain has special importance because it is a putative binding site for Wnt ligands (Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. & Nusse, R. (1996) Nature 382, 225–30). The FRP CRD is 30–42% identical to the CRD of the other FZ proteins.

In addition to the plasma membrane-anchored FZ proteins and FRP, three other molecules have been described which also possess a FZ CRD motif. An alternatively spliced isoform of mouse collagen XVIII was the first such protein to be reported (Rehn, M. & Pihlajaniemi, T. (1995) J Biol Chem 270, 4705-11). The two other molecules, mouse SDF5 (Shirozu, M., Tada, H., Tashiro, K., Nakamura, T., Lopez, N. D., Nazarea, M., Hamada, T., Sato, T., Nakano, T. & Honjo, T. (1996) Genomics 37, 273-280) and human FRZB (Hoang, B., Moos, M., Jr., Vukicevic, S. & Luyten, F. P. (1996) J Biol Chem 271, 26131-26137), resemble FRP in that each consists of ~300 amino acid residues, including a signal peptide, CRD near its NH2-terminus and a hydrophilic COOH-terminal moiety. FRP and SDF5 have 58% identities in their CRDs, while FRP and FRZB are only 32% identical in this region. Elsewhere, these molecules are only 15-20% identical. Thus, FRP, SDF5 and FRZB may constitute a subfamily of small, FZ-related proteins that lack the seven transmembrane motif responsible for anchoring FZ proteins to the plasma membrane and are presumably secreted.

Figures 1 and 2 and 8 provide illustrations of these protocols and results. Figure 1 consists of a series of panels. Panel (B) shows a representation of human FRP cDNA clones. Overlapping clones HS1 and HS8 are shown above a diagram of the complete coding sequence and the adjacent 5' and 3' untranslated regions. The coding region is boxed; the open portion corresponds to the signal sequence. Untranslated regions are represented by a line. Selected restriction sites are indicated. Panel (C) shows the predicted FRP amino acid sequence (standard single-letter code). The peptide sequence obtained from the purified protein is underlined. Double-underlined sequences were used to generate oligonucleotide probes for screening of the M426 cDNA library. The putative signal sequence is italicized. The large shaded region is the cysteine-rich domain homologous to CRD's in members of the FZ family. The small shaded region is the lysine-rich segment that fulfills the criteria for a consensus hyaluronic acid-binding sequence. The dashed underlining denotes two potential asparagine-linked glycosylation sites.

Figure 8 shows the nucleic acid sequence which encodes a FRP polypeptide. At ~nucleotide 340 we denote with an asterisk a site in the molecule where we observe an insert of the sequence "CAG" in some constructs. This would result in an insert of a single amino acid residue (alanine) in the putative signal peptide sequence without altering any of the remaining amino acid sequence. This may result from alternative splicing or possibly a sequencing artifact.

Figure 2 provides comparisons of the CRDs of FRP and other members of the FZ family. Solid black shading highlights identities present in human FRP and any

other FZ family member. The consensus sequence indicates residues present in at least eight of the sixteen FZ or FZ-related proteins. Double asterisks denote the ten invariant cysteine residues; single asterisks indicate other invariant residues. hFRP, human FZ-related protein; hFZ (Zhao, Z., Lee, C., Baldini, A. & Caskey, C. T. (1995) Genomics 27, 370-3); hFZ5 (Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N.A. & Nathans, J. (1996) J Biol Chem 271, 4468-76); mFZ3-mFZ8 (Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N.A. & Nathans, J. (1996) J Biol Chem 271, 4468-76); rFZ1 and rFZ2 (Chan, S. D., Karpf, D. B, Fowlkes, M. E., Hooks, M., Bradley, M. S., Vuoung, V., Bambino, T., Liu, M. Y., Arnaud, C. D., Strewler, G. J. & et al. (1992) J Biol Chem 267, 25202-7); dFZ (Vinson, C. R., Conover, S. & Adler, P. N. (1989) Nature 338, 263-4); dFZ2 (Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J. & Nusse, R. (1996) Nature 382, 225-30); cFZ (Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N.A. & Nathans, J. (1996) J Biol Chem 271, 4468-76); mCOL, mouse collagen XVIII (Rehn, M. & Pihlajaniemi, T. (1995) J Biol Chem 270, 4705-11); hFRZB (Hoang, B., Moos, M., Jr., Vukicevic, S. & Luyten, F. P. (1996) J Biol Chem 271, 26131 7); mSDF5 (Shirozu, M., Tada, H., Tashiro, K., Nakamura, T., Lopez, N. D., Nazarea, M., Hamada, T., Sato, T., Nakano, T. & Honjo, T. (1996) Genomics 37, 273-280).

EXAMPLE 3

Expression of the FRP Gene.

Northern and Southern Blot Analysis.

RNA from cell lines was isolated, transferred to nitrocellulose filters, and hybridized with labeled probes as previously described (Finch, P. W., Rubin, J. S., Miki, T., Ron, D. & Aaronson, S. A. (1989) Science 245, 752 5). Northern blots containing approximately 2 μg of poly A+RNA isolated from a variety of different organs were purchased from Clontech (Palo Alto, CA). Labeled probes were hybridized in Express Hyb hybridization solution (Clontech) according to the manufacturer's protocol. The FRP NotI-SmaI cDNA fragment and human β-actin cDNA probe provided by Clontech were ^{32}P-labeled with random hexamers and used at a concentration of $1-2 \times 10^6$ cpm/ml (specific activity $>8\times10^8$ cpm/μg DNA).

Southern blotting was performed as previously described (Kelley, M. J., Pech, M., Seuanez, H. N., Rubin, J. S., O'Brien, S. J. & Aaronson, S. A. (1992) Proc Natl Acad Sci U S A 89, 9287-91), except for variation in formamide concentration during

hybridization, as noted in the text. FRP cDNA probes were ^{32}P-labeled with the nick-translation kit from Amersham.

FRP Gene is Expressed in Multiple Organs and Cell Types.

Using the 1081-bp NotI-SmaI fragment of HS1 (Fig. 1B) as probe, a single 4.4 kb transcript was detected in polyA+ RNA from several human organs (Fig. 3). In adult tissues, the highest level of expression was observed in heart, followed by kidney, ovary, prostate, testis, small intestine and colon. Lower levels were seen in placenta, spleen and brain, while transcript was barely detectable in skeletal muscle and pancreas. No hybridization signal was evident in mRNA from lung, liver, thymus or peripheral blood leukocytes. In poly A+ RNA from a small sample of human fetal organs, the 4.4 kb transcript was highly represented in kidney, at moderate levels in brain, barely detectable in lung, and undetectable in liver.

Northern analysis of total RNA from various human cell lines demonstrated the 4.4 kb transcript and, occasionally, additional faint bands not further analyzed (Fig. 3, extreme right panel). While the transcript was detected in RNA from embryonic lung (M426 and WI-38) and neonatal foreskin (AB1523) fibroblasts, it was not observed in a sample of adult dermal fibroblasts (501T). In addition to fibroblasts, the transcript was seen in RNA from primary keratinocytes, indicating that expression was not limited to cells of mesenchymal origin. Considering that the cumulative size of the initial overlapping FRP cDNAs was only 2.8 kb, detection of a 4.4 kb transcript reinforced the suggestion that the cDNAs were generated by internal priming at adenine-rich regions. An illustration of the sequence of this larger transcript is provided in Figure 8.

Developmental Expression of FRP

Northern blot analysis of samples from human organs indicates that the FRP transcript was expressed at many sites, and the level of expression varied in embryonic and adult tissues.

Finch, et al., P.N.A.S. 94: 6770-6775 (1997). To assess its pattern of expression and potential role in development, we investigated the distribution of FRP transcript in mouse embryos by *in situ* hybridization analysis. A 144-bp mouse FRP cDNA fragment was generated by RT-PCR, using total RNA from NIH/3T3 fibroblasts as template. After subcloning into pGEM3Zf(-), ^{35}S-labeled sense and antisense riboprobes were prepared for hybridization. These experiments suggest that FRP transcript is present in embryos from 8.5 days to birth, with highest overall levels of expression observed at 12.5-14.5 days. At this peak period, expression was

documented in discrete portions of the central nervous system, gastrointestinal tract, genitourinary system, lining of the abdominal cavity, heart and developing vertebrae. Transcript was present either in mesenchymal or epithelial cells, depending on the location. The transient aspect of FRP spatiotemporal distribution reinforced the idea that its expression is regulated during development. Like the Wnt proteins, FRP participates in processes that govern embryonic development.

Detection of FRP in other species.

To determine whether the FRP gene was present in other species, genomic DNAs from various sources were fully digested with EcoRI and hybridized with an NcoI–SmaI cDNA fragment (Fig. 1B) under varying conditions of stringency (Fig. 5). Multiple bands were observed under highly stringent conditions (50% formamide) in DNA from human, rhesus monkey, mouse and chicken. With moderate stringency (35% formamide), no additional fragments were seen in the DNA from these species but fragments were detected in *Xenopus* DNA. No hybridization signal was observed with DNA from *Drosophila* or yeast (*S. cervisiae*) in these experiments. At low stringency (20% formamide), the background was too high to detect specific signals. These results strongly suggested that the FRP gene is highly conserved among vertebrates. Although these experiments did not detect an FRP homolog in the invertebrates, the existence of such homologs was not rigorously excluded, due to the limitations of the method.

Southern blotting performed either with the NotI–NcoI cDNA fragment (Fig. 1B) or with synthetic oligonucleotide probes corresponding to different portions of the FRP coding sequence, hybridized to subsets of genomic fragments detected with the NcoI–SmaI probe. This finding and the lack of additional bands detected only under relaxed conditions (Fig. 5) indicated that highly related FRP–like sequences are not present in the human genome. Thus, the multiple genomic fragments hybridizing to the FRP cDNA in Southern blots are likely to reflect the presence of several exons in the hFRP gene.

Figures 3 and 5 provide an illustration of these protocols and results. Figure 3 shows FRP mRNA expression in normal human adult and embryonic tissues, and in cultured cells. Blots containing approximately 2 μg of poly A+ RNA from each of the indicated tissues or 10 μg of total RNA from different human cell lines were probed with radiolabeled FRP and β–actin cDNA fragments, as described in the Methods. The position of DNA size markers, expressed in kb, is indicated at the left of the tissue blots; the position of 28S and 18S ribosomal RNA is shown at the left of the cell blot.

Figure 5 shows a southern blot analysis of FRP genomic sequences in different species. After fractionation by agarose gel electrophoresis and transfer to filters, Eco-RI-digested genomic DNAs were hybridized in the presence of either 50% or 35% formamide. Specimens were from the following species: λ, lambda phage; H, human; M^k, rhesus monkey; M^o, mouse; C, chicken; X, *Xenopus laevis*; D, *Drosophila melanogaster*; Y, yeast (*S. cerevisiae*).

EXAMPLE 4

Chromosomal Localization of FRP.

A 4.1 kb *FRP* genomic fragment obtained from a human fibroblast genomic DNA library (Stratagene) was labeled with biotin or digoxigenin and used as a probe for *in situ* hybridization to locate the *FRP* gene in chromosomal preparations of methotrexate-synchronized normal peripheral human lymphocyte cultures. The conditions for hybridization, detection of fluorescent signal, digital-image acquisition, processing and analysis were as previously described (Zimonjic, D. B., Popescu, N. C., Matsui, T., Ito, M. & Chihara, K. (1994) Cytogenet Cell Genet 65, 184-5). The identity of the chromosomes with specific signal was confirmed by rehybridization using a chromosome 8-specific probe, and the signal was localized on G-banded chromosomes.

Using a fluorescent-labeled 4.1 kb genomic fragment containing a portion of the FRP coding sequence, *in situ* hybridization revealed a single locus at chromosome 8p11.1-12 (Fig. 4). This site may be near the putative locus of the *hFZ3* gene, based on homology with the location of *mFz3* in the mouse genome (Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N.A. & Nathans, J. (1996) J Biol Chem 271, 4468-76). Radiation hybrid analysis yielded results consistent with the fluorescent *in situ* hybridization analysis. Significantly, the chromosomal locus of the FRP gene is compatible with that of a tumor suppressor gene associated prostate, colon, and non small cell lung carcinoma. Information on FRP genomic structure and its relationship to defects in these malignancies is a material avenue of research in this field.

Figure 4 provides an illustration of these protocols and results. Figure 4 shows the chromosomal localization of the FRP gene by fluorescent *in situ* hybridization. To localize the FRP gene, one hundred sets of metaphase chromosomes were analyzed. In eighty metaphases, a double fluorescent signal was observed with the FRP genomic problem in 8p11.2-12 on both chromosome homologs (left panel). The identity of the chromosomes was confirmed by hybridization with a probe specific for chromosomes 8 (right panel).

EXAMPLE 5

Biosynthetic Studies of FRP.

For biosynthetic studies, M426 cells grown in T–25 flasks were incubated for 30 min. in methionine–free DMEM in the presence or absence of 50μg/ml heparin (bovine lung, Sigma; when present, heparin was included in all subsequent media), which was subsequently replaced with medium containing ^{35}S–methionine (1 mCi/5 ml per dish). After 30 min., the radioactive medium was removed, and monolayers washed with medium containing unlabeled methionine, then incubated for varying intervals in fresh nonradioactive medium. At the specified times, the conditioned media and cell lysates were collected and processed as previously described (Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H. Taylor, W. G., Cech, A. C., Hirshfield, D. W., Wong, J., Miki, T., Finch, P. W. & et al. (1991) Proc Natl Acad Sci U S A 88, 415–9). Immunoprecipitations were performed with a rabbit polyclonal antiserum (100 μg/ml) raised against a synthetic peptide corresponding to FRP amino acid residues 41–54, in the presence or absence of competing peptide (50 μg/ml). Immune complexes adsorbed to GammaBind (Pharmacia) were pelleted by centrifugation and washed; labeled proteins were resolved by SDS/PAGE and detected by autoradiography.

Figure 6 provides an illustration of these results. Figure 6 shows the biosynthesis of FRP in M426 cells. A pulse–chase experiment was performed with metabolically labeled cells incubated either in the absence or presence of heparin. Proteins were immunoprecipitated from cell lysates (C.L.) or conditioned medium (C.M.) with FRP peptide antiserum in the absence or presence of competing peptide, and resolved in a 10% polyacrylamide SDS gel. Cells and media were harvested 1, 4 or 20 hours after a 30 min labeling period. Lanes 1–24 are labeled at the bottom. The protein band corresponding to FRP is indicated by an arrow. The position of molecular mass markers is shown at the left.

<u>FRP is Secreted, but Primarily Cell–Associated in the Absence of Exogenous Heparin.</u>

To study the synthesis and processing of FRP protein, a pulse–chase experiment was performed with ^{35}S–methionine labeled M426 cells either in the absence or presence of added heparin. As shown in Fig. 6, a 36 kDa protein band was specifically immunoprecipitated with antiserum raised against a synthetic peptide corresponding to a portion of the FRP NH2–terminal sequence. In the absence of soluble heparin, after either 1 hour (lanes 1 and 5) or 4 hours (lanes 9 and 13) FRP

was much more abundant in the cell lysate than in the conditioned medium. However, after 20 hours, the amount of FRP protein in the medium (lane 21) was comparable to that which remained cell–associated (lane 17). At this last time point, the combined band intensity in the two compartments had decreased relative to that observed earlier, suggesting significant protein turnover during the experiment. Moreover, after 20hr the FRP–specific signal appeared as a doublet, providing additional evidence of proteolysis. In the presence of soluble heparin (50μg/ml), most of the FRP was detected in the medium at all three time points (compare lanes 3 and 7, 11 and 15, 19 and 23). Heparin also appeared to stabilize FRP, as the band intensity was stronger when heparin was present, and there was no evidence of partial proteolysis. Interestingly, others have shown that heparin can release Wnt–1 from the cell surface in a similar manner (Papkoff, J. & Schryver, B. (1990) *Mol Cell Biol* **10**, 2723–30; Bradley, R. S. & Brown, A. M. (1990) *Embo J* **9**, 1569–75; Reichsman, F., Smith, L. & Cumberledge, S. (1996) *J Cell Biol* **135**, 819–27). Taken together, our results demonstrate that FRP is secreted, although it tends to remain cell–associated and relatively susceptible to degradation unless released into the medium by soluble heparin.

<u>FRP binds to hyaluronic acid.</u>

As shown by the amino acid sequence in figure 1C, FRP contains a lysine–rich segment that fulfills the criteria for a consensus hyaluronic acid–binding sequence (Yang, B., Yang, B. L., Savani, R. C. & Turley, E. A. (1994) Embo J 13, 286–96). Figure 9 shows the binding of FRP to biotinylated hyaluronic acid in a transblot assay under either nonreducing (–) or reducing (+) conditions (Yang, B, Zhang, L., and Turley, E.A. (1993) J. Bio. Chem. 268, 8617–8623; Hardwick, C., Hoare, K., Owens, R. Hohn, H.P., Hook, M., D., Cripps and Turley, E.A. (1992) J. Cell Biol. 117, 1343–1350). Further, Figure 10 shows the competition of BHA binding to FRP by various proteoglycans, C.S. is chondroitin sulfate, H.A. is hyaluronic acid, H.A. oligo is hyaluronic acid oligosaccaride. The Ab control consists of a western blot of FRP with rabbit polyclonal antiserum raised against FRP synthetic peptide.

Figures 9 and 10 provide an illustration of these results. Figure 9 shows the binding of FRP to biotinylated hyaluronic acid in a transblot assay under either nonreducing (–) or reducing (=) conditions. Figure 10 shows the competition of BHA binding to FRP by various proteoglycans, C.S. is chondroitin sulfate, H.A. is hyaluronic acid, H.A. oligo is hyaluronic acid oligosaccaride. The Ab control consists of a western blot of FRP with rabbit polyclonal antiserum raised against FRP synthetic peptide.

EXAMPLE 6

Modulation of *Xenopus* Development By FRP.

Wnt-1, wg, Xwnt-3a and Xwnt-8 plasmids were used as described (McMahon, A. P. & Moon, R. T. (1989) *Development* **107 Suppl**, 161-7; Chakrabarti, A., Matthews, G., Colman, A. & Dale, L. (1992) *Development* **115**, 355-69; Wolda, S. L., Moody, C. J. & Moon, R. T. (1993) *Dev Biol* **155**, 46-57; Smith, W. C. & Harland, R. M. (1991) *Cell* **67**, 753-65). The FRP NaeI-SalI cDNA fragment, which includes the full coding sequence, was subcloned into the StuI and XhoI sites of pCS2+ (Turner, D. L. & Weintraub, H. (1994) Genes Dev 8, 1434-47). All mRNAs for injection were synthesized as capped transcripts *in vitro* with SP6 RNA polymerase (Ambion Megascript Kit). Embryo preparation and staging were performed as described (He, X., Saint-Jeannet, J. P., Woodgett, J. R., Varmus, H. E. & Dawid, I. B. (1995) Nature 374, 617-22). Transcripts were injected into the two blastomeres near the equatorial midline region at the 4-cell stage.

<u>FRP Antagonizes Wnt Action in *Xenopus* Embryo Assay.</u>

Because FRP possesses a potential binding site for Wnt molecules and appears to partition among cellular compartments like Wnt-1, it seemed possible that FRP might modulate the signaling activity of Wnt proteins. We envisioned two alternatives: FRP might antagonize Wnt function by binding the protein and blocking access to its cell surface signaling receptor, or FRP might enhance Wnt activity by facilitating the presentation of ligand to the FZ receptors, analogous to the action of soluble interleukin 6 receptors (Kishimoto, T., Taga, T. & Akira, S. (1994) Cell 76, 253-62).

To test these possibilities, we examined the effect of FRP on Wnt-dependent dorsal axis duplication during *Xenopus* embryogenesis. Previous studies have demonstrated that microinjection of mRNA encoding certain Wnt molecules, such as mouse Wnt-1, Wg, XWnt-8 or XWnt-3a, into early *Xenopus* embryos can induce the formation of an ectopic Spemann organizer and, subsequently, duplication of the dorsal axis (McMahon, A. P. & Moon, R. T. (1989) *Development* **107 Suppl**, 161-7; Chakrabarti, A., Matthews, G., Colman, A. & Dale, L. (1992) *Development* **115**, 355-69; Wolda, S. L., Moody, C. J. & Moon, R. T. (1993) *Dev Biol* **155**, 46-57; Smith, W. C. & Harland, R. M. (1991) *Cell* **67**, 753-65; Moon, R. T., Christian, J. L., Campbell, R. M., McGrew, L., DeMarais, A., Torres, M., Lai, C. J., Olson, D.J. & Kelly, G. M. (1993) *Dev Suppl*, 85-94; Sokol, S., Christian, J. L., Moon, R. T. & Melton, D. A.

(1991) *Cell* **67**, 741–52). In addition, it has been reported that FRZB is a secreted antagonist of Wnt signaling expressed in the Spemann organizer (Leyns, L., Bouwmeester, T., Kim, S. H., Piccolo, S. & De Robertis, E. M. (1997) *Cell* **88**, 747–756, Wang, S., Krinks, M., Lin, K., Luyten, F. P. & Moos, M. J. (1997) *Cell* **88**, 757–766).

Figure 7 provides an illustration of these results. Figure 7 shows the dorsal axis duplication in *Xenopus* embryos in response to varying combinations of Wnt and FRP transcripts. The total number of embryos injected in two to four independent experiments is indicated by the value of n; each bar represents the percentage of axis duplication; the solid portion within each bar represents the percentage of extensive duplication, which is defined by the presence of the cement gland and at least one eye in the duplicated axis. The amount of mRNA injected per embryo is shown below the bars.

As illustrated in Fig. 7, injection of suboptimal doses of Wnt–1, Wg, or XWnt–8 mRNA into embryos induced partial or complete duplication in at least 75% of the animals. Suboptimal doses were used to enable the detection of enhancement of the axis duplication phenotype, if the role of FRP was to facilitate Wnt signaling. However, when similar quantities of FRP and Wnt RNA were coinjected, the incidence and extent of axial duplication were significantly reduced (Fig. 7). The effect was dose–dependent, as the number of animals with an abnormal phenotype was even lower when the relative amount of FRP RNA was increased five– to ten–fold. Injection of FRP RNA alone at a higher dose (100 pg) into the dorsal side of the embryo did not affect the endogenous dorsal axis formation.

Surprisingly, FRP was much less effective in antagonizing XWnt–3a, suggesting a degree of specificity regarding interactions with different members of the Wnt family. The Wnt signaling pathway is thought to proceed through suppression of the activity of glycogen synthase kinase–3, a cytoplasmic serine–threonine kinase (Miller, J. R. & Moon, R. T. (1996) Genes Dev 10, 2527–39). Axis duplication induced by a dominant–negative, kinase–inactive mutant of glycogen synthase kinase–3β (He, X., Saint–Jeannet, J. P., Woodgett, J. R., Varmus, H. E. & Dawid, I. B. (1995) *Nature* **374**, 617–22; Dominguez, I., Itoh, K. & Sokol, S.Y. (1995) *Proc Natl Acad Sci U S A* **92**, 8498–502; Pierce, S. B. & Kimelman, D. (1995) *Development* **121**, 755–65) was not affected by FRP, consistent with the assumption that FRP directly interferes with Wnt signaling at the cell surface, not by indirectly interfering with a late step in the Wnt signaling pathway.

EXAMPLE 7

Expression and Purification of Recombinant FRP.

As disclosed in detail below, recombinant FRP has been produced in a stable mammalian expression system involving Madin-Darby canine kidney (MDCK) cells. One of the advantages of eukaryotic expression is the reliability of disulfide bond formation and associated protein folding, which are likely to be important in the synthesis of the secreted cysteine-rich FRP protein. In contrast, preliminary experiments with prokaryotic expression yielded protein that appeared to be heterogeneous with respect to the folding of disulfide bonds. MDCK cells were transfected by standard calcium phosphate precipitation methodology with a pcDNA vector (Invitrogen) containing the FRP coding sequence. Following G418 selection of transfected cells, immunoblot analysis of conditioned medium from a mass culture revealed the presence of recombinant FRP-crossreactive protein. The amount of FRP in the medium from FRP-transfected MDCK cells appeared to be far greater than quantities produced by cell lines naturally expressing FRP.

As discussed in detail below, the MDCK/FRP culture was expanded into T-175 flasks and a series of pilot experiments conducted to develop a scheme for the purification of recombinant protein. Once the cells reached confluence, serum-containing growth medium was removed, the monolayer was washed twice with phosphate-buffered saline, and then serum-free medium was added. After 72 hours, the culture fluid was harvested and serum-containing medium was added to the flasks. Subsequently, the monolayer was again washed and serum-free medium introduced for another 72 hour period of conditioning. This process was repeated four or five times with the same monolayer cultures. The conditioned medium was promptly concentrated by ultrafiltration at 4°C in a stirred chamber with a 10-kDa molecular mass cutoff (Amicon). Immunoblot analysis confirmed that FRP protein was present in the retentate, and its concentration was markedly increased compared to that of the starting material. The retentate was fractionated by heparin-TSK high performance liquid chromatography (HPLC).

Figure 12 provides an illustration of these results. Figure 12 shows recombinant FRP. (A) Preparation of FRP protein. The FRP coding sequence was subcloned into pcDNA3.1(+) and transfected into MDCK cells by standard calcium phosphate precipitation methodology. Following selection, transfected cells were grown to confluence and switched to serum-free medium. After 72 hours, conditioned medium was collected, concentrated by ultrafiltration and fractionated by heparin-TSK HPLC. At least 90% of the protein did not bind to resin equilibrated in 0.05M phosphate/0.15M NaCl/pH 7.4. After eluting the less tightly bound protein with 0.5M NaCl (data not shown), a modified linear gradient of increasing [NaCl] was

used to recover the remaining protein. (B) Immunoblotting with FRP peptide antiserum. Ten μl aliquots of selected 1 min fractions from heparin-TSK chromatography (indicated by bar in panel A) were resolved by 12% SDS-PAGE, transferred to Immobilon filters, blotted with FRP amino-terminal peptide antiserum (Finch, et al., P.N.A.S. 94: 6770-6775 (1997)) and analyzed by chemiluminescence. Position of molecular mass markers (kDa) is shown at left. (C) Silver-staining of FRP-containing fractions from heparin-TSK chromatography. Five μl aliquots of indicated fractions were subjected to 12% SDS-PAGE and silver-stained (BioRad kit). Position of molecular mass markers (kDa) is shown at left.

Most protein did not bind to the resin, which had been equilibrated at neutral pH in isotonic buffer. Following a stepwise increase of NaCl concentration to 0.5M, the remaining protein was eluted with a modified linear gradient of increasing [NaCl] (Figure 12A). FRP was detected by western blotting of aliquots from the major, overlapping protein peaks which eluted with 1.1-1.4M NaCl (Figure 12B). Silver-staining of proteins resolved by SDS-PAGE demonstrated that the peak fractions only contained bands corresponding in size to immunoreactive FRP (Figure 12C). Based on optical density, the estimated yield of FRP protein was 0.5-1.0 mg from two liters of conditioned medium (~50-100 times more than the original, naturally occurring source).

Detailed Recombinant FRP Recombinant Expression Protocols.

Vectors and constructs included pcDNA3.1(+) with both the full-length human FRP coding sequence as well as site directed mutants of full-length FRP, in which substitutions (Asn to Gln) were made at either or both of the Asn-linked glycosylation sites. Additional vectors included pcDNA3.1(-)/Myc-His C designed to link Myc and His tags to the carboxy-terminus of recombinant protein of the full-length human FRP coding sequence as well as three truncated FRP derivatives, each lacking a varying amount of the carboxy-terminal portion of the full-length protein; sequences span: 1-171, 1-221, and 1-242.

Constructs were transfected into MDCK cells by standard calcium phosphate methodology, and cultures were subjected to selection with antibiotic (G418, 0.5 mg/ml). Transfected mass culture was expanded, ultimately grown in the absence of G418 (growth medium is DMEM plus 10% fetal bovine serum). Clonal lines were isolated from the mass culture of FRP/MDCK transfectants and screened for elevated FRP expression by immunoblot analysis of culture fluid.

To generate conditioned medium, cells were grown to confluence in T175 flasks (alternatives known in the art also should be suitable, such as cell factories or

microcarriers in bioreactors). Medium in these experiments was switched from DMEM plus 10% fetal bovine serum to serum-free DMEM. After 2 or 3 days, conditioned medium was harvested and optionally another round of serum-free DMEM was added, to be harvested, typically after another 3 days. As many as 10 collections could be made from a single monolayer. In some instances, cells were cycled from serum-free to serum containing medium, before switching back to serum-free medium for subsequent collection of conditioned medium

Medium was filtered through a 0.45 micron membrane prior to concentration by ultrafiltration. Optionally medium was clarified by centrifugation before filtration. For ultrafiltration, conditioned medium was concentrated in a stainless steel, Amicon model 2000 stirred cell with a YM-10 membrane (10 kD molecular mass cutoff) at 4°C. Typically volumes of 1-2 liters were reduced to 45-90 ml. Concentrates were snap-frozen and stored in freezer.

Purification of FRP.

As discussed above, wild type FRP was purified with heparin affinity chromatography. Details of chromatography varied, from linear NaCl gradient with heparin-HPLC column to stepwise NaCl elution with Pharmacia Hi-Trap heparin column. FRP elutes with approximately 1.0 M NaCl.

FRP derivatives containing a Myc-His tag were purified with nickel resin (such as a Pharmacia Hi-Trap His column), typically being eluted with 50 or 100 mM imidazole solution. FRP-containing fractions were identified by immunoblotting and silver stain analysis following SDS-PAGE (12% polyacrylamide). Fractions were snap-frozen and stored in freezer.

EXAMPLE 8

FRP and FRP Binding Partner Interaction Assays.

A substantial body of data strongly suggests that Frizzled molecules function as receptors or components of receptors for Wnt proteins. (See e.g., He, et al., Science 275:1652-1654 (1997.)) Deletional analysis indicated that the cysteine-rich domain (CRD) is responsible for conferring Wnt-binding or Wnt-dependent signaling to biological systems. Bhanot, et al., Nature 382:225-230 (1996). Because FRP contains a FZ-type CRD and is able to antagonize Wnt-dependent duplication of the dorsal axis in the *Xenopus* embryo assay (Finch, et al., P.N.A.S. 94:6770-6775 (1997), FRP is likely to be a receptor for a subset of Wnt family members. To test the

hypothesis that FRP inhibits Wnt activity by binding Wnt proteins, the assays to elucidate the interaction of FRP with FRP binding proteins were designed.

Experimental systems to study Wnt binding and other interactions are problematic for a number of reasons. For years, Wnt receptor analysis has been hampered by the insolubility of Wnt proteins, which tend to remain associated with cell surfaces or extracellular matrix. Bradley, et al., EMBO J. 9:1569–1575 (1990). This property has impeded efforts to purify Wnts for tracer labeling and specific, sensitive receptor–ligand binding studies. Moreover, because vertebrates produce at least eight different Frizzleds Wang, et al., J. Biol. Chem. 271:4468–4476 (1996), six secreted Frizzled–related proteins (see e.g., Salic, et al., Development 124:4739–4748 (1997) and fifteen Wnts (Cadigan, et al., Genes Dev. 11:3286–3305 (1997), any cell is likely to endogenously express one or more molecules that could influence Wnt–Frizzled binding [not to mention proteoglycans, which also affect Wnt binding and activity. (See e.g., Häcker, et al., Development 124:3565–3573 (1997).] This would undoubtedly complicate the interpretation of experiments involving ectopic expression of any component of the putative Wnt–FZ binding complex.

As an alternative, cell–free systems to study the binding of FRP and Wnts were developed. These systems have the advantage of simplicity, as the profile of endogenous Frizzled/FRP/Wnt expression would not be an issue. Moreover, experiments now can be performed with purified recombinant FRP. One model system disclosed in detail below makes use of an ELISA type of format. Microtiter wells are first coated with purified FRP, and then blocked with a large excess of bovine serum albumin (BSA). To minimize the problems associated with solubilizing Wnts, initial studies focused on studying FRP binding to Wingless (Wg) because an expression system is available that releases Wg into conditioned medium. Van Leeuwen, et al., Nature 368:342–344 (1994). Although expression of a heat shock–Wg construct in transfected S2 insect cells results in only a small fraction of recombinant Wg in the media, it is sufficient for binding studies and obviates the need for detergents or other agents to extract the protein from cell surfaces or extracellular matrix. Medium from the heat shock–Wg cells or S2 vector controls is incubated in microtiter wells that have either been coated with FRP and blocked with BSA or only blocked with BSA. After washing, the wells are sequentially incubated with antibody to Wg, secondary antiserum (against the Wg antibody) conjugated to alkaline phosphatase, and *p*–nitrophenol phosphate. The last reagent is a substrate for the phosphatase; the development of yellow color is a measure of the Wg protein retained in the wells.

Figure 13 provides an illustration of these results. Figure 13 illustrates the binding of FRP and Wg. (A) FRP-Wg interaction in ELISA format. Falcon 96-well microtiter plates were coated with recombinant human FRP at 100 ng/well (FRP 100) or left blank (FRP 0) prior to blocking with 4% bovine serum albumin. Subsequently wells were incubated with serial dilutions of conditioned medium from S2 cells expressing Wg via a heat shock promoter (Wg) or medium from control S2 cells (S2). Following washing, wells were incubated sequentially with a rabbit polyclonal antiserum against Wg, goat anti-rabbit antiserum conjugated to alkaline phosphatase, and *p*-nitrophenol phosphate. Color development, monitored at 405 nm, was indicative of Wg binding in the well. Each data point is the mean ± standard deviation of triplicate measurements; when not shown, deviation was smaller than size of symbol. (B) Immunoblotting of Wg. Samples of conditioned medium used in (A) were concentrated in Centricon-10 devices, resolved by 8% SDS-PAGE, transferred to Immobilon filters, blotted with Wg monoclonal antibody and analyzed by chemiluminescence. The arrow indicates band corresponding to Wg. Position of molecular mass markers is shown at left.

As shown in Figure 13A, wells coated with 100 ng of FRP display specific and highly reproducible binding of Wg that varies with the dilution of conditioned medium. A small amount of non-specific binding is observed when relatively concentrated Wg-containing samples are incubated in wells that have not been coated with FRP. No background signal is seen in wells coated with FRP and incubated with medium from control S2 cells. This is consistent with the fact that control cells do not express detectable levels of Wg protein (Figure 13B). Taken together, these data provide strong evidence that FRP is capable of binding Wg. These assays are described in detail below.

FRP-Wingless Standard ELISA Assay.

Wells of ELISA plate were coated with purified, recombinant FRP. After blocking with BSA, conditioned medium containing Wingless (Wg) was introduced. Following appropriate incubation period, Wg-containing medium was removed and bound Wg was detected by sequential addition of antibody to Wg, secondary antibody conjugated to alkaline phosphatase and p-nitrophenylphosphate. Amount of yellow color in well, determined by ELISA reader (spectrophotometer measuring absorbance at 405 nm), was a measure of bound Wg. Either the amount of FRP used to coat wells or the dilution of Wg medium (or control medium) could be varied to generate additional quantitative information about the interaction between FRP and Wg.

FRP coating of the wells was accomplished by diluting FRP in 0.02% sodium azide/PBS; and add 50 ul to each well. 100–300 ng FRP/well provides optimal results. The plate was then incubated at 37°C for 2 hours in a moist environment. In this assay, FRP was not added to the first lane, which serves as a blank. Blocking was accomplished by removing the FRP solution from the wells and adding 100 ul of a 4% BSA in sodium azide/PBS (no need to wash) and incubating at 37°C for 2 hours. Washing was accomplished by washing the wells 5 times with TAPS (0.05% Tween 20 and 0.02% NaN_3/PBS). Wells were filled with squeeze bottle, and then blotted against paper towel.

Wg binding was accomplished diluting Wg–containing or control media in diluent solution (1% BSA in TAPS), and add 50 ul to each well and then incubating at room temperature overnight. The wells were then washed 5 times as disclosed above. The primary antibody was then added by diluting the anti–Wg antibody (mouse monoclonal) in diluent solution to 1:1000, and add 50 ul to each well and incubating at 37°C for 2 hours. The wells were then washed 5 times as disclosed above.

The secondary antibody was added by diluting this secondary antibody (goat anti–mouse–alkaline phosphatase conjugate) in diluent solution (using a conjugate from TAGO Inc. cat #4650 at a 1:400 dilution); add 50 ul to each well and incubating at 37°C or 2 hours. The wells were then washed 5 times as disclosed above. Substrate was prepared by dissolving the substrate, p–nitrophenolphosphate (Sigma cat# 2640) in carbonate buffer (1mM $MgCl_2$ / 0.1M Na_2CO_3 pH 9.8) to a final concentration of 2mg/ml. 65 ul of this substrate was then added to each well and read OD at 405 nm.

<u>Competition Assay</u>

Similar to standard assay discussed above, except Wg–containing medium is preincubated with varying concentrations of FRP or related protein to assess the ability of these proteins to bind Wg. This is manifested by a reduction in Wingless binding to the FRP–coated ELISA well. Typically preincubation is performed at room temperature for 1 hour.

A related variation of the ELISA involves preincubation of Wg medium with proteoglycan such as heparin to assess its effect on subsequent binding to FRP coated wells. Medium can be preincubated with heparin and FRP simultaneously. As an alternative, heparin and/or FRP (or FRP–related proteins) can be added to the medium in the ELISA well without prior incubation. Figure 14 provides an illustration of these results. Specifically, Figure 14 illustrates how soluble FRP (ng of FRP/50 ul of Wg containing medium) influences Wg binding to bound FRP in ELISA format.

Preparation of Conditioned Medium from Drosophila S2 Cells
– Control S2 and Cells Transfected with Heat Shock–Wingless Construct

Growth medium utilized in this assay was Schneider's Drosophila Medium (Gibco cat # 11720–034) + supplements. The medium for conditioning was Shields and Sang M3 Insect Medium (Sigma cat# S3652).

The Recovery of frozen cells appears to be the time when cells are most fragile (aside from recovery period, the cells are quite hardy). A key point is to avoid diluting cells until they clearly are thriving. Specifically, an ampule of ~10 million cells in a T–25 flask with final volume of 5–6 ml medium consisting of: Schneider's medium, 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 ug/ml); incubated at 26°C. Periodically, a small amount (1–2 ml) of fresh medium, prewarmed to room temperature was added to the solution, allowing the cell population to become quite dense (turbid) prior to subculture.

For the initial subculture, the cell suspension was transferred with pipette to a T–75 flask containing 20–25 ml of the above medium; typically suspension was diluted from 1:2 to 1:10, or perhaps even greater. Because some cells will remain adherent to the original T–25 flask, fresh medium was added to it to maintain a viable culture. Cells typically grow well at this point. For the subsequent subculture, dense (turbid) cultures were split, usually 1:10, into T–175 flasks for experiments. Cultures become dense and ready for use in ~1 week.

Heat shock protocol:

The heat shock protocol was carried out by the following steps:

1. Incubate culture at 37°C for 50 minutes;
2. Transfer culture to room temp (26°C), and incubate for 40 minutes;
3. Pellet cells at low speed (3000 rpm, 5 minutes in standard lab bench centrifuge);
4. Resuspend pellets 3 times with serum–free Shields and Sang M3 Insect Medium, 20–40 ml/wash and obtain cell count (alternatively one can determine cell count with aliquot of sample during step 1), (typically, cell number is determined after the second resuspension by removing an aliquot, diluting it 5–fold and measuring cell count with a hemacytometer);
5. After the third wash, resuspend cells in Shields and Sang medium at a concentration of 25 million cells/ml and incubate for 5 hours at 26°C; and
6. Pellet cells as before and collect supernatant for use as conditioned medium.

FRP–Wg Crosslinking

FRP–Wg interactions in a cell–free setting were studied first, as variables are more easily controlled in such systems than in cellular assays. The crosslinking analysis may be extended to cellular systems. In particular, one can assess FRP binding to epitope–tagged, Wnt family members expressed in appropriate host cells such as NIH/3T3 fibroblasts. Crosslinked complexes consisting of ^{125}I–FRP and Wnt protein were immunoprecipitated with antibodies directly against the epitope tag.

A method for detection of FRP–Wg complexes by crosslinking analysis is as follows.

I. The iodination reaction can be accomplished by reacting 10 ug of purified recombinant FRP and 1 milliCurie of $Na^{125}I$ with chloramine–T for 1 minute at room temperature (additional details essentially as described in Bottaro DP et al. J Biol. Chem 265: 12767–12770, (1990)).

The ^{125}I–FRP can be isolated by heparin–Sepharose chromatography. Specifically, tracer was eluted with phosphate buffer (pH7.4) containing 1.0 M NaCl and 1 mg/ml bovine serum albumin (BSA). Certain iodinated FRP derivatives can be recovered on desalting columns (containing resins such as G10) that serve to separate protein tracer from free sodium iodide. Tracer was stored frozen and subjected to not more than a few rounds of freeze–thawing prior to use.

II. Binding of tracer to Wg (all performed at room temperature) can be undertaken as follows. Typically ~ 1 microCurie (though amount could vary), of ^{125}I–FRP, was incubated with medium from Wg–expressing S2 cells (or control S2 cells) in a final volume of 50 ul for 40 minute. Varying amounts of additional reagents, such as heparin and/or unlabeled FRP, were added in some experiments. After binding period, crosslinking reagent bis(sulfosuccinimidyl) suberate (BS^3) was added at a final concentration of 1 mM and incubation continued for 20 minutes. The crosslinking reaction was terminated by the addition of Tris–HCl and glycine (final concentrations were 1 mM and 20 mM, respectively).

III. Detection of crosslinked complexes was undertaken by incubating all or most of the reaction mixture with antibody directed against Wg overnight at 4°C. Protein G–coupled resin (~50 ul slurry of GammaBind, Pharmacia) was then added along with buffer A (50 mM HEPES pH 7.4, 5 mM EDTA, 50 mM NaCl, 1% Triton X–100, 6 mM $Na_4P_2O_7$, 50 mM NaF, 0.35 mg/ml PMSF,10 ug/ml aprotinin and 10 ug/ml leupeptin) to bring final volume to ~0.5 ml. The reaction mixture was incubated for 1 hour at 4°C in a rotary shaker. Typically a monoclonal antibody to Wg was used for immunoprecipitation, final concentration of 10 ug/ml (Brook et al., Science 273: 1373–1377 (1996)). After incubation, immune complexes were pelleted

by centrifugation in microfuge (3 min at 14,000 rpm). Pellets were washed 3 times, each with 1 ml of buffer A

Laemmli sample buffer was added to pellets, samples boiled for 4 minutes and proteins resolved by SDS-PAGE (8% polyacrylamide). In some instances, aliquots were removed from reaction mixture prior to addition of antibody and processed for electrophoresis. Gels were fixed (in 20%methanol, 10% acetic acid, 70% water) for 30 minutes at room temperature, dried and exposed to X-ray film at $-70^{\circ}C$ for autoradiography.

Figures 15 and 16 illustrate these ^{125}I-FRP-Wg Crosslinking reactions under different experimental conditions. Briefly, Figure 15 shows the effects of varying the concentration of heparin in crosslinking reactions between ^{125}I-FRP and Wg, with the crosslinked molecules being immunoprecipitated with an anti-Wg monoclonal antibody and separated by gel electrophoresis. In this assay, varying amounts of heparin (Fisher, porcine intestinal) were incubated with ^{125}I-FRP (approximately 1 microCurie) and conditioned medium from Wg-expressing or control S2 cells at room temperature for 40 min. After a subsequent incubation with BS3 crosslinking agent, the reaction was quenched and the mixture was subjected to immunoprecipitation with monoclonal antibody to Wg. Precipitates were resolved by SDS-PAGE and labeled protein detected by autoradiography of dried gels.

Figure 16 shows the effects of varying the concentration of unlabelled FRP or FRP derivatives in crosslinking reactions between ^{125}I-FRP and Wg, with the crosslinked molecules being immunoprecipitated with an anti-Wg monoclonal antibody and separated by gel electrophoresis. Briefly, varying concentrations of unlabeled FRP or FRP derivatives were incubated with ^{125}I-FRP, conditioned medium from Wg-expressing or control S2 cells and heparin at 1 ug/ml. After a subsequent incubation with BS3 crosslinking agent, the reaction was quenched and the mixture was subjected to immunoprecipitation with monoclonal antibody to Wg. Precipitates were resolved by SDS-PAGE and labeled protein detected by autoradiography of dried gels.

EXAMPLE 9

Modulation of Embryonic Kidney Cell Tubulogenesis By FRP.

Induction of tubulogenesis in culture of isolated metanephric mesenchyme was assessed. This organ culture system was described in an article by Karavanova ID et al. (Development 122: 4159-4167, 1996). In brief, kidneys were removed from F344 rat embryos 13 days post coitum. Metanephric mesenchyme was separated from ureteric bud by enzyme treatment, and cultured on collagen-coated filters. Tissue

incubated with serum–free conditioned medium from a rat ureteric bud cell line (RUB1) supplemented with basic FGF and TGFα was induced to differentiate into epithelial tubular structures corresponding to nephrons.

Remarkably, if cultures treated with RUB1 conditioned medium, basic FGF and TGFα also received purified recombinant FRP (5 ug/ml) the induction of tubular structures was completely inhibited. Interestingly, the mesenchymal cells did not die; they even appeared to increase in number and the cultures grew larger during the 3 day incubation period. This result, an increase in condensed mesenchyme but an apparent failure to differentiate into epithelial cells and subsequent failure to form tubular structures, was observed in vivo in mice that were targeted for loss of Wnt 4 expression (Stark K et al. Nature 372: 679–683, 1994). Moreover, other Wnt family members are expressed in kidney and may participate in this process of differentiation and morphogenesis (see Karavanova et al. 1996). Thus, this preliminary result provides additional support for the idea that FRP can function as a soluble antagonist of Wnt activity. On a more elementary level, it demonstrates that purified, recombinant FRP has biological activity.

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: THE GOVERNMENT OF THE UNITED STATES, as represented by the Secretary, Department of Health and Human Services

(ii) TITLE OF THE INVENTION: HUMAN FRP AND FRAGMENTS THEREOF INCLUDING METHODS OF USING THEM

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 3100 Norwest Center, 90 South Seventh Street
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(D) STATE: Minnesota
(E) COUNTRY: USA
(F) ZIP: 55402-4131

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 29-May-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/050,417
(B) FILING DATE: 29-May-1997
(C) APPLICATION NUMBER: 60/050,495
(D) FILING DATE: 23-June-1997

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Wood, William J.
(B) REGISTRATION NUMBER: P-42,236
(C) REFERENCE/DOCKET NUMBER: 11613.13WOI1

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 310-445-1140
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4497 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2114 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 314 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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LNKNCHAGTQ VFLCSLFAPV CLDRPIYPCR WLCEAVRDSC EPVMQFFGFY
WPEMLKCDKF PEGDVCIAMT PPNATEASKP QGTTVCPPCD NELKSEAIIE
HLCASEFALR MKIKEVKKEN GDKKIVPKKK KPLKLGPIKK KDLKKLVLYL
KNGADCPCHQ LDNLSHHFLI MGRKVKSQYL LTAIHKWDKK
NKEFKNFMKK
MKNHECPTFQ SVFK

What is claimed is:

1. A polypeptide comprising a isolated human FRP comprising in part a Wnt binding domain.

2. The polypeptide of claim 1 wherein the Wnt binding domain is shown in the large shaded region of Figure 1C.

3. A molecule including therein the polypeptide of claim 1.

4. The polypeptide of claim 1, further comprising a hyaluronic acid binding sequence.

5. The polypeptide of claim 1 having the amino acid sequence as shown in Figure 1.

6. The polypeptide of claim 1 joined to a non–FRP binding molecule.

7. The polypeptide of claim 6, wherein the non–FRP binding molecule is a protein.

8. The polypeptide of claim 7, wherein the protein is an immunoglobulin molecule.

9. The polypeptide of claim 7 joined to a detectable label.

10. The polypeptide of claim 9, wherein the detectable label is selected from the group consisting of radioactive isotopes, enzymes, fluorophores or chromophores.

11. A polypeptide comprising a isolated human FRP comprising in part a Wnt binding domain and a hyaluronic acid binding domain.

12. The polypeptide of claim 11, wherein the Wnt binding domain is shown in the large shaded region of Figure 1C.

13. The polypeptide of claim 11, wherein the hyaluronic acid binding domain is shown in the small shaded region of Figure 1C.

14. The polypeptide of claim 11 comprising the amino acid sequence as shown in Figure 1

15. The polypeptide of claim 11 joined to a detectable label.

16. The polypeptide of claim 15, wherein the detectable label is selected from the group consisting of radioactive isotopes, enzymes, fluorophores or chromophores.

17. An isolated polynucleotide encoding a human FRP polypeptide.

18. The polypeptide of claim 17, wherein the polypeptide includes a Wnt binding domain.

19. The polynucleotide of claim 17 comprising a nucleotide sequence coding for an amino acid sequence as shown in Figure 1.

20. The polynucleotide molecule of claim 19, wherein the Wnt binding domain encodes amino acids 57–166 of Figure 1.

21. The polynucleotide of claim 17 wherein the polynucleotide sequence is codon optimized for a specific host cell.

22. The polynucleotide of claim 17 joined to a detectable label.

23. The polynucleotide of claim 17, wherein the detectable label is selected from the group consisting of radioactive isotopes, enzymes and chromophores.

24. A polynucleotide probe capable of hybridizing with the polynucleotide of claim 17.

25. The polynucleotide of claim 17, wherein the polynucleotide is DNA.

26. The polynucleotide of claim 25, wherein the DNA is cDNA.

27. The polynucleotide of claim 17, wherein the polynucleotide is RNA.

28. The polynucleotide of claim 27, wherein the RNA is mRNA.

29. A vector comprising the polynucleotide of claim 17.

30. The vector of claim 29, wherein the polynucleotide is operably linked to at least one control sequence(s) capable of being recognized by a host cell transformed with the vector.

31. The vector of claim 30, wherein at least one control sequence is a cytomegalovirus promoter.

32. A host cell comprising the vector of claim 30.

33. The host cell of claim 32, wherein the host cells are Madin–Darby canine kidney cells.

34. A process for producing FRP polypeptide comprising culturing the host cell of claim 32 under conditions such that the FRP polypeptide is produced.

35. A FRP polypeptide produced by the method of claim 34.

36. A FRP antisense oligonucleotide comprising a polynucleotide which is complimentary to an mRNA encoding human FRP.

37. An isolated FRP specific polypeptide comprising a F_{ab} fragment from an antibody capable of specifically binding to a FRP polypeptide.

38. The isolated FRP specific polypeptide of claim 37, wherein the polypeptide comprises an isolated antibody.

39. The FRP specific polypeptide of claim 38 wherein the antibody is a polyclonal, monoclonal or chimeric antibody.

40. A non–human transgenic animal whose somatic and germ cells contain a

transgene comprising human FRP.

41. The transgenic animal of claim 40, wherein the transgene effects a reduction in FRP gene expression.

42. The transgenic animal of claim 40, wherein the transgene effects an increase in FRP gene expression.

43. The transgenic animal of claim 40, wherein the transgene is operably linked to at least one control sequence(s) capable of being recognized by the transgenic animal.

44. The transgenic animal of claim 43, wherein at least one control sequence is the mouse mammary tumor virus long terminal repeat.

45. A method of assaying a sample for a polynucleotide encoding a FRP polypeptide, comprising:

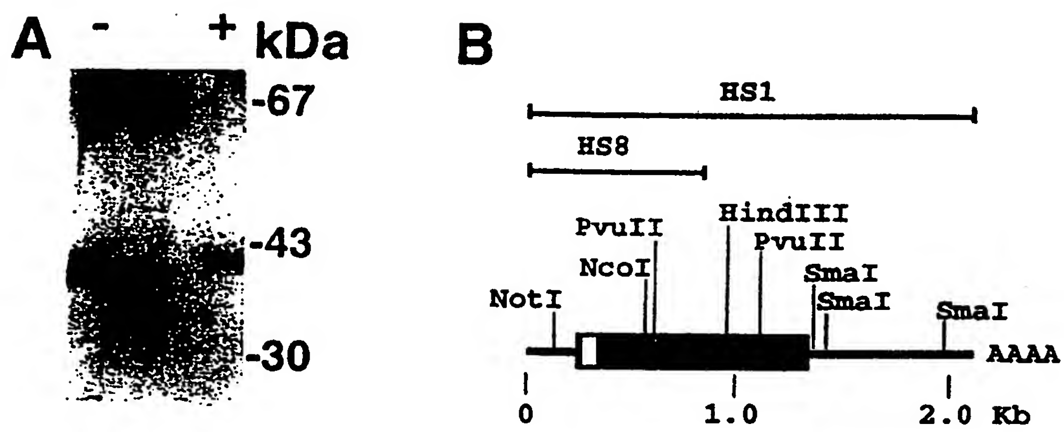
detecting the presence or absence of the FRP polynucleotide in the sample utilizing the polynucleotide probe of claim 24.

46. A method of assaying a sample for a FRP polypeptide comprising:

detecting the presence or absence of the FRP polypeptide in the sample utilizing an isolated FRP specific polypeptide which includes a F_{ab} fragment from an antibody capable of specifically binding to the FRP polypeptide.

47. A method of detecting the presence of a FRP binding protein in a sample comprising contacting the biological sample with FRP so that the FRP binds the FRP binding protein and determining the presence of an FRP–FRP binding protein complex.

48. The method of claim 47, wherein the FRP binding protein comprises the drosophila Wingless molecule.



C

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LDRPIYPCRWLCEAVRDSCEPVMQFFGFYWPEMLKCDKFP
        170       180       190       200
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        210       220       230       240
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```

Figure 1A & 1B & 1C

```
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              *          *           **                            *
              *          *                                         *
CONSENSUS     C-PI-..IPL C--I-YN-T- MPNLLGH--Q --AGLEVHQF -PLV---CSP

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mSDF5         DTKKFLCSLF APVCLDDLD. ETICPCHSLC MQVKDRCAPV MSAFGFPWPD
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hFZ           RLR..CEHFP RHG..AEQIC  150
hFZ5          RMS..CDRLP VLGRDAEVLC  147
mFZ3          DME..CSRFP D........C  133
mFZ4          TLN..CSKFP PQN.DHNHMC  158
mFZ6          ELE..CNRLP H........C  129
mFZ7          RLR..CENFP VHG..AGEIC  160
mFZ8          RMR..CDRLP EQG.NPDTLC  148
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rFZ2          RLR..CEHFP RHG..AEQIC  155
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cFZ           VIR..CDKFP LEN.NREKMC  139
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hFRZB         NLA..CEELP VYDR...GVC  147
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Figure 2

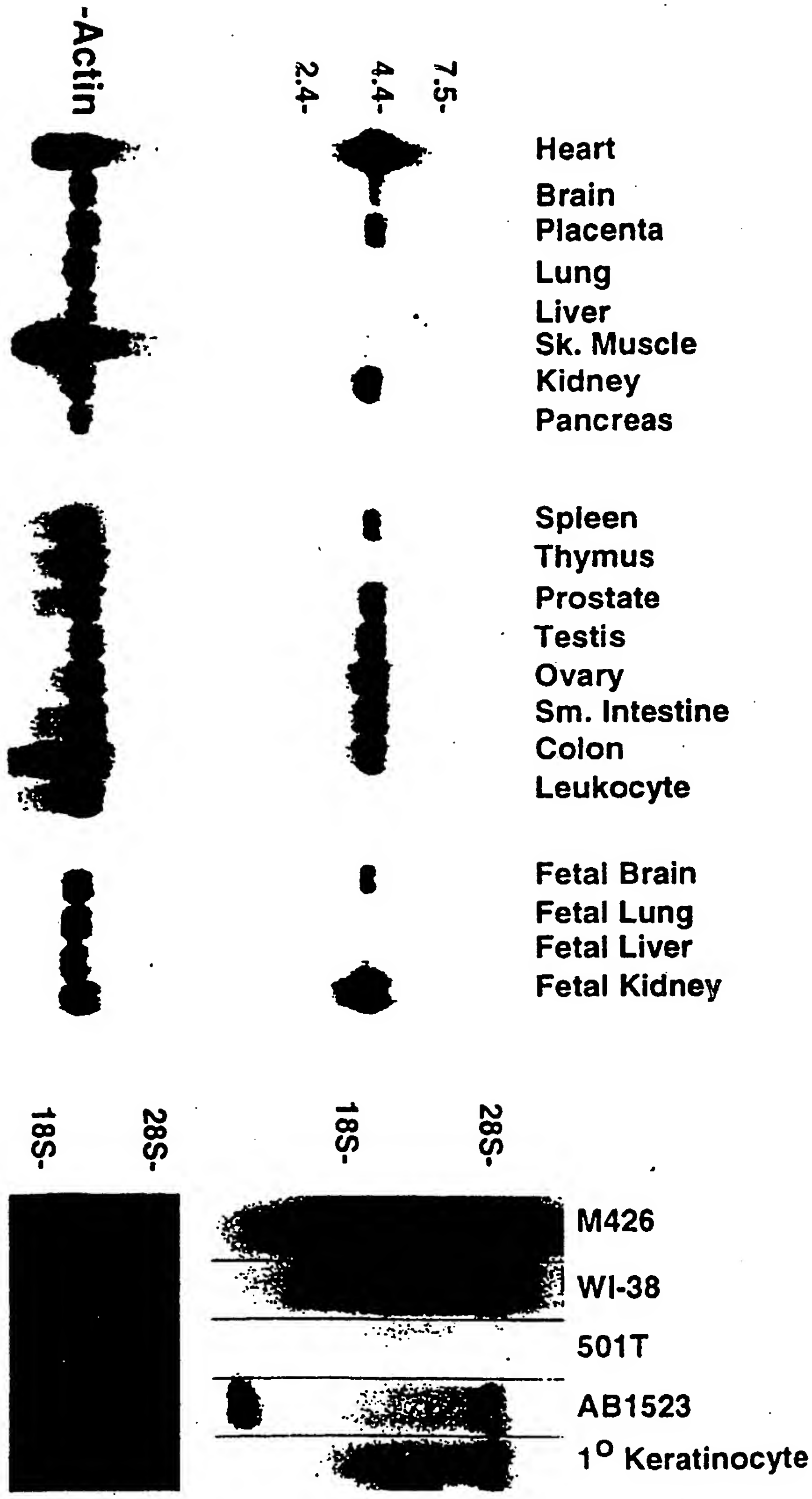
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Figure 3

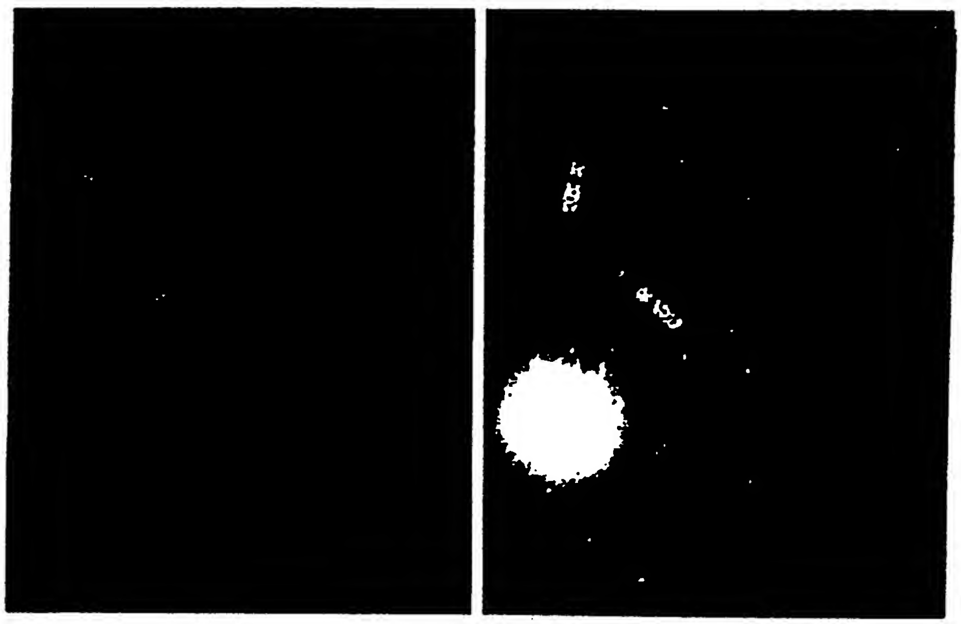


Figure 4

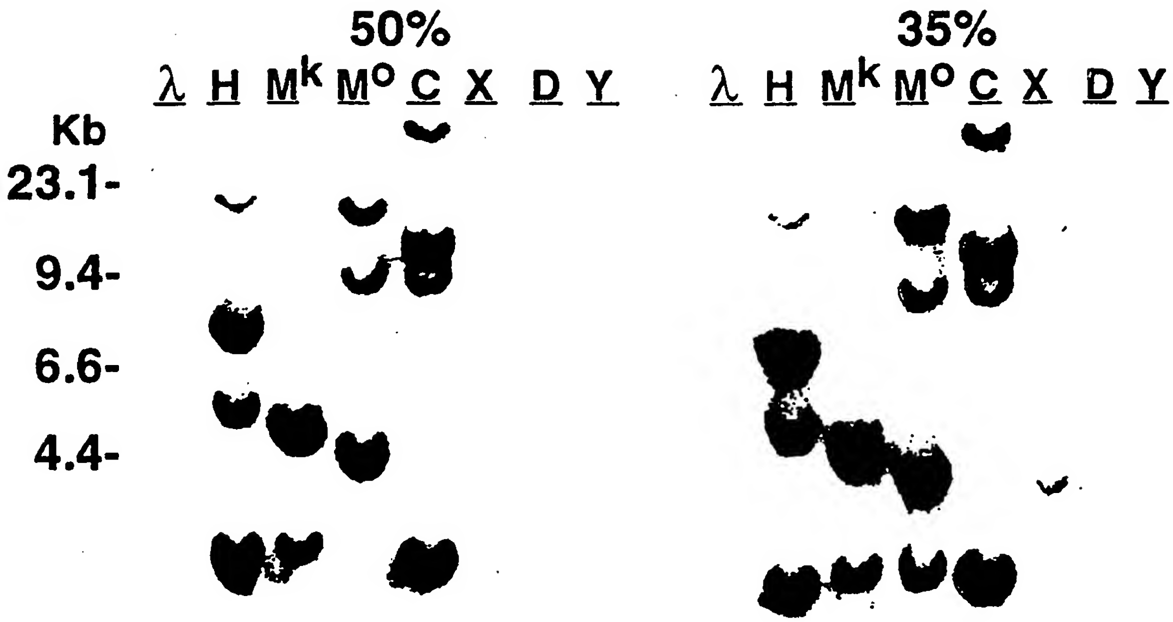
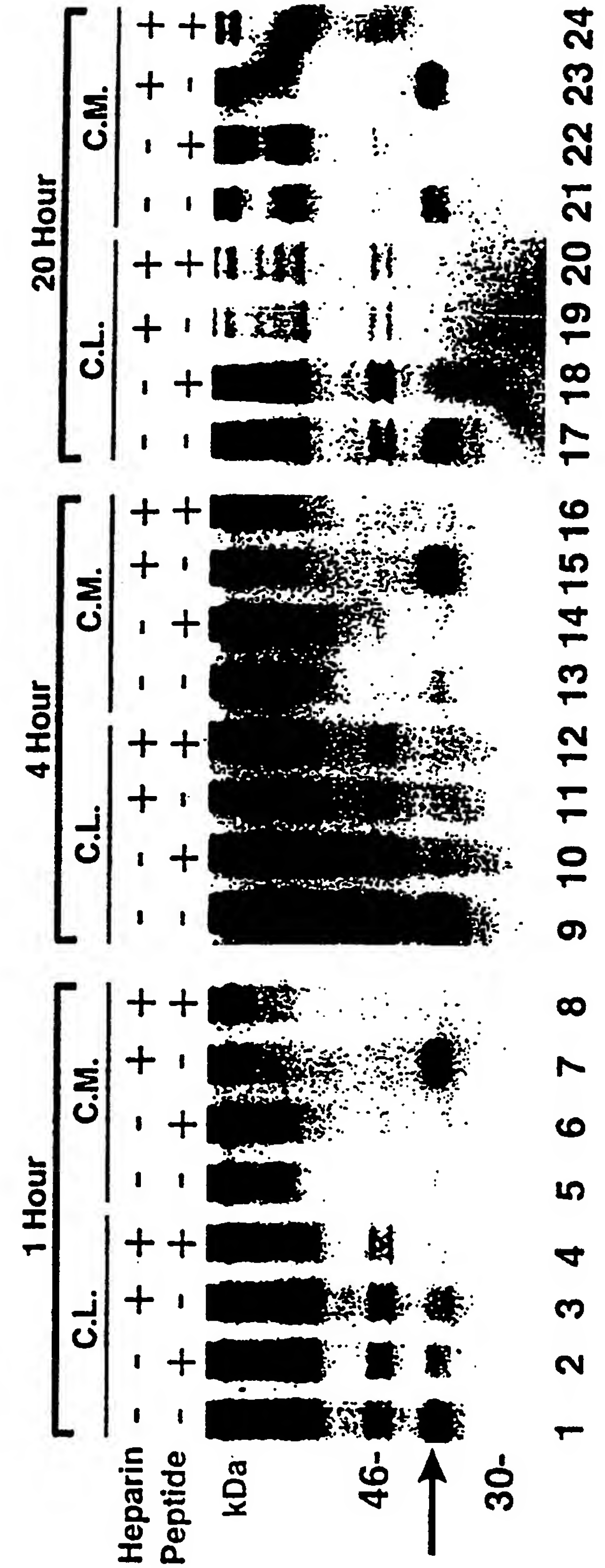


Figure 5


1 Hour
4 Hour
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C.L.
C.M.
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kDa
46-
30-
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 6

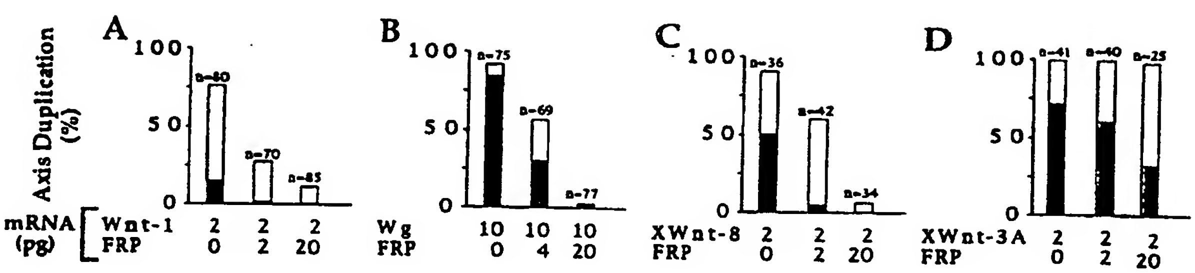
A
B
C
D
Axis Duplication (%)
100
50
0
mRNA (pg)
Wnt-1 2 2 2
FRP 0 2 20
Wg 10 10 10
FRP 0 4 20
XWnt-8 2 2 2
FRP 0 2 20
XWnt-3A 2 2 2
FRP 0 2 20

Figure 7

FRP Sequence (HS1), GenBank Submission

FRP from 1 to 2075:

10 20 30 40 50 60 70
cctgcagcct ccggagtcag tgccgcgcgc ccgccgcccc gcgccttcct gctcgccgca cctccgggag

80 90 100 110 120 130 140
ccggggcgca cccagcccgc agcgccgcct ccccgcccgc gccgcctccg accgcaggcc gagggccgcc

150 160 170 180 190 200 210
actggccggg gggaccgggc agcagcttgc ggccgcggag ccgggcaacg ctggggactg cgcctttgt

220 230 240 250 260 270 280
ccccggaggt ccctggaagt ttgcggcagg acgcgcgcgg ggaggcggcg gaggcagccc cgacgtcgcg

290 300 310 320 330 340 350
gagaacaggg cgcagagccg gcatgggcat cgggcgcagc gaggggggcc gccgcgggg*c cctgggcgtg

360 370 380 390 400 410 420
ctgctggcgc tgggcgcggc gcttctggcc gtgggctcgg ccagcgagta cgactacgtg agcttccagt

430 440 450 460 470 480 490
cggacatcgg cccgtaccag agcgggcgct tctacaccaa gccacctcag tgcgtggaca tccccgcgga

500 510 520 530 540 550 560
cctgcggctg tgccacaacg tgggctacaa gaagatggtg ctgcccaacc tgctggagca cgagaccatg

570 580 590 600 610 620 630
gcggaggtga agcagcaggc cagcagctgg gtgcccctgc tcaacaagaa ctgccacgcc gggacccag

640 650 660 670 680 690 700
gtcttcctct gctcgctctt cgcgcccgtc tgcctggacc ggcccatcta cccgtgtcgc tggctctgcg

710 720 730 740 750 760 770
aggccgtgcg cgactcgtgc gagccggtca tgcagttctt cggcttctac tggcccgaga tgcttaagtg

780 790 800 810 820 830 840
tgacaagttc ccggaggggg acgtctgcat cgccatgacg ccgcccaatg ccaccgaagc ctccaagccc

850 860 870 880 890 900 910
caaggcacaa cggtgtgtcc tccctgtgac aacgagttga aatctgaggc catcattgaa catctctgtg

920 930 940 950 960 970 980
ccagcgagtt tgcactgagg atgaaaataa aagaagtgaa aaaagaaaat ggcgacaaga agattgtccc

990 1000 1010 1020 1030 1040 1050
caagaagaag aagcccctga agttggggcc catcaagaag aaggacctga agaagcttgt gctgtacctg

1060 1070 1080 1090 1100 1110 1120
aagaatgggg ctgactgtcc ctgccaccag ctggacaacc tcagccacca cttcctcatc atgggccgca

Figure 8a

1130 1140 1150 1160 1170 1180 1190
aggtgaagag ccagtacttg ctgacggcca tccacaagtg ggacaagaaa aacaaggagt tcaaaaacttc

1200 1210 1220 1230 1240 1250 1260
atgaagaaaa tgaaaaacca tgagtgcccc acctttcagt ccgtgtttaa gtgattctcc cgggggcagg

1270 1280 1290 1300 1310 1320 1330
gtggggaggg agcctcgggt ggggtgggag cggggggac agtgcccggg aacccgtggt cacacacacg

1340 1350 1360 1370 1380 1390 1400
cactgccctg tcagtagtgg acattgtaat ccagtcggct tgttcttgca gcattcccgc tccctttccc

1410 1420 1430 1440 1450 1460 1470
tccatagcca cgctccaaac cccagggtag ccatggccgg gtaaagcaag ggccatttag attaggaagg

1480 1490 1500 1510 1520 1530 1540
tttttaagat ccgcaatgtg gagcagcagc cactgcacag gaggaggtga caaaccattt ccaacagcaa

1550 1560 1570 1580 1590 1600 1610
cacagccact aaaacacaaa aagggggatt gggcggaaag tgagagccag cagcaaaaac tacattttgc

1620 1630 1640 1650 1660 1670 1680
aacttgttgg tgtggatcta ttggctgatc tatgcctttc aactagaaaa ttctaatgat tggcaagtca

1690 1700 1710 1720 1730 1740 1750
cgttgttttc aggtccagag tagtttcttt ctgtctgctt taaatggaaa cagactcata ccacacttac

1760 1770 1780 1790 1800 1810 1820
aattaaggtc aagcccagaa agtgataagt gcagggagga aaagtgcaag tccattatct aatagtgaca

1830 1840 1850 1860 1870 1880 1890
gcaaagggac caggggagag gcattgcctt ctctgcccac agtctttccg tgtgattgtc tttgaatctg

1900 1910 1920 1930 1940 1950 1960
aatcagccag tctcagatgc ccccaaagttt cggttcctat gagcccgggg catgatctga tccccaagac

1970 1980 1990 2000 2010 2020 2030
atgtggaggg gcagcctgtg cctgcctttg tgtcagaaaa aggaaaccac agtgagcctg agagagacgg

2040 2050 2060 2070
cgattttcgg gctgagaagg cagtagtttt caaaacacat agtta

* We have observed an insert of sequence 'cag' at this site in some cDNA constructs. This would result in an insert of a single amino acid residue, alanine, in the putative signal peptide sequence without altering any of the remaining amino acid sequence. This may result from alternative splicing, but we have not excluded the possibility of a sequencing artifact. We hope to resolve this matter soon. The numbering scheme in the above figure corresponds to the sequence lacking 'cag.'

Figure 8b

```
             10        20        30        40        50        60        70        80        90       100
     1234567890123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890

0001 CTCGGCCGTAGGAGCCCCGCGCACTCCAGCCCTGCAGCCTCCGGAGTCAGTGCCGCGCGCCCGCCGCCCCGCGCCTTCCTGCTCGCCGCACCTCCGGGAG 0100
0101 CCGGGGCGCACCCAGCCCGCAGCGCCGCCTCCCCGCCCGCGCCGCCTCCGACCGCAGGCCGAGGGCCGCCACTGGCCGGGGGACCGGGCAGCAGCTTGC 0200
0201 GGCCGCGGAGCCGGGCAACGCTGGGGACTGCGCCTTTTGTCCCCGGAGGTCCCTGGAAGTTTGCGGCAGGACGCGGGCGGGGAGGCGGCGGAGGCAGCCC 0300
0301 CGACGTCGCGGAGAACAGGGCGCAGAGCCGGCATGGGCATCGGGCGCAGCGAGGGGGGCCGCCGCGGGGCAGCCCTGGGCGTGCTGCTGGCGCTGGGCGC 0400
0401 GGCGCTTCTGGCCGTGGGCTCGGCCAGCGAGTACGACTACGTGAGCTTCCAGTCGGACATCGGCCCGTACCAGAGCGGGCGCTTCTACACCAAGCCACCT 0500
0501 CAGTGCGTGGACATCCCCGCGGACCTGCGGCTGTGCCACAACGTGGGCTACAAGAAGATGGTGCTGCCCAACCTGCTGGAGCACGAGACCATGGCGGAGG 0600
0601 TGAAGCAGCAGGCCAGCAGCTGGGTGCCCCTGCTCAACAAGAACTGCCACGCCGGCACCCAGGTCTTCCTCTGCTCGCTCTTCGCGCCCGTCTGCCTGGA 0700
0701 CCGGCCCATCTACCCGTGTCGCTGGCTCTGCGAGGCCGTGCGCGACTCGTGCGAGCCGGTCATGCAGTTCTTCGGCTTCTACTGGCCCGAGATGCTTAAG 0800
0801 TGTGACAAGTTCCCCGAGGGGGACGTCTGCATCGCCATGACGCCGCCCAATGCCACCGAAGCCTCCAAGCCCCAAGGCACAACGGTGTGTCCTCCCTGTG 0900
0901 ACAACGAGTTGAAATCTGAGGCCATCATTGAACATCTCTGTGCCAGCGAGTTTGCACTGAGGATGAAAATAAAAGAAGTGAAAAAAGAAAATGGCGACAA 1000
1001 GAAGATTGTCCCCAAGAAGAAGAAGCCCCTGAAGTTGGGGCCCATCAAGAAGAAGGACCTGAAGAAGCTTGTGCTGTACCTGAAGAATGGGGCTGACTGT 1100
1101 CCCTGCCACCAGCTGGACAACCTCAGCCACCACTTCCTCATCATGGGCCGCAAGGTGAAGAGCCAGTACTTGCTGACGGCCATCCACAAGTGGGACAAGA 1200
1201 AAAACAAGGAGTTCAAAAACTTCATGAAGAAAATGAAAAACCATGAGTGCCCCACCTTTCAGTCCGTGTTTAAGTGATTCTCCCGGGGGCAGGGTGGGGA 1300
1301 GGGAGCCTCGGGTGGGGTGGGAGCGGGGGGACAGTGCCCCGGGAACCCGGTGGGTCACACACACGCACTGCGCCTGTCAGTAGTGGACATTGTAATCCA 1400
1401 GTCGGCTTGTTCTTGCAGCATTCCCGCTCCCTTCCCTCCATAGCCACGCTCCAAACCCCAGGGTAGCCATGGCCGGGTAAAGCAAGGGCCATTTAGATTA 1500
1501 GGAAGGTTTTTAAGATCCGCAATGTGGAGCAGCAGCCACTGCACAGGAGGAGGTGACAAACCATTTCCAACAGCAACACAGCCACTAAAACACAAAAAGG 1600
1601 GGGATTGGGCGGAAAGTGAGAGCCAGCAGCAAAAACTACATTTTGCAACTTGTTGGTGTGGATCTATTGGCTGATCTATGCCTTTCAACTAGAAAATTCT 1700
1701 AATGATTGGCAAGTCACGTTGTTTTCAGGTCCAGAGTAGTTTCTTTCTGTCTGCTTTAAATGGAAACAGACTCATACCACACTTACAATTAAGGTCAAGC 1800
1801 CCAGAAAGTGATAAGTGCAGGGAGGAAAAGTGCAAGTCCATTATGTAATAGTGACAGCAAAGGGACCAGGGGAGAGGCATTGCCTTCTCTGCCCACAGTC 1900
1901 TTTCCGTGTGATTGTCTTTGAATCTGAATCAGCCAGTCTCAGATGCCCCAAAGTTTCGGTTCCTATGAGCCCGGGGCATGATCTGATCCCCAAGACATGT 2000
2001 GGAGGGGCAGCCTGTGCCTGCCTTTGTGTCAGAAAAAGGAAACCACAGTGAGCCTGAGAGAGACGGCGATTTTCGGGCTGAGAAGGCAGTAGTTTTCAAA 2100
2101 ACACATAGTTAAAAAAGAAACAAATGAAAAAAATTTTAGAACAGTCCAGCAAATTGCTAGTCAGGGTGAATTGTGAAATTGGGTGAAGAGCTTAGGATTC 2200
2201 TAATCTCATGTTTTTTCCTTTTCACATTTTTAAAAGAACAATGACAAACACCCACTTATTTTTCAAGGTTTTAAAACAGTCTACATTGAGCATTTGAAAG 2300
2301 GTGTGCTAGAACAAGGTCTCCTGATCCGTCCGAGGCTGCTTCCCAGAGGAGCAGCTCTCCCCAGGCATTTGCCAAGGGAGGCGGATTTCCCTGGTAGTGT 2400
2401 AGCTGTGTGGCTTTCCTTCCTGAAGAGTCCGTGGTTGCCCTAGAACCTAACACCCCCTAGCAAAACTCACAGAGCTTTCCGTTTTTTTCTTTCCTGTAAA 2500
2501 GAAACATTTCCTTTGAACTTGATTGCCTATGGATCAAAGAAATTCAGAACAGCCTGCCTGTCCCCCCGCACTTTTTACATATATTTGTTTCATTTCTGCA 2600
2601 GATGGAAAGTTGACATGGGTGGGGTGTCCCCATCCAGCGAGAGAGTTTCAAAAGCAAAACATCTCTGCAGTTTTTCCCAAGTACCCTGAGATACTTCCCA 2700
2701 AAGCCCTTATGTTTAATCAGCGATGTATATAAGCCAGTTCACTTAGACAACTTTACCCTTCTTGTCCAATGTACAGGAAGTAGTTCTAAAAAAAATGCAT 2800
2801 ATTAATTTCTTCCCCCAAAGCCGGATTCTTAATTCTCTGCAACACTTTGAGGACATTTATGATTGTCCCTCTGGGCCAATGCTTATACCCAGTGAGGATG 2900
2901 CTGCAGTGAGGCTGTAAAGTGGCCCCCTGCGGCCCTAGCCTGACCCGGAGAAAGGATGGTAGATTCTGTTAACTCTTGAAGACTCCAGTATGAAAATCAG 3000
3001 CATGCCCGCCTAGTTACCTACCGGAGAGTTATCCTGATAAATTAACCTCTCACAGTTAGTGATCCTGTCCTTTTAACACCTTTTTTGTGGGGTTCTCTCT 3100
3101 GACCTTTCATCGTAAAGTGCTGGGGACCTTAAGTGATTTGCCTGTAATTTTGGATGATTAAAAAATGTGTATATATATATTAGCTAATCAGAAATATTCTAC 3200
3201 TTCTCTGTTGTCAAACTGAAATTCAGAGCAAGTTCCTGAGTGCGTGGATCTGGGTCTTAGTTCTGGTTGATTCACTCAAGAGTTCAGTGCTCATACGTAT 3300
3301 CTGCTCATTTTGACAAAGTGCCTCATGCAACCGGGCCCTCTCTCTGCGGCAGAGTCCTTAGTGGAGGGGTTTACCTGGAACATAGTAGTTACCACAGAAT 3400
3401 ACGGAAGAGCAGGTGACTGTGCTGTGCAGCTCTCTAAATGGGAATTCTCAGGTAGGAAGCAACAGCTTCAGAAAGAGCTCAAAATAAATTGGAAATGTGA 3500
3501 ATCGCAGCTGTGGGTTTTACCACCGTCTGTCTCAGAGTCCCAGGACCTTGAGTGTCATTAGTTACTTTATTGAAGGTTTTAGACCCATAGCAGCTTTGTC 3600
3601 TCTGTCACATCAGCAATTTCAGAACCAAAAGGGAGGCTCTCTGTAGGCACAGAGCTGCACTATCACGAGCCTTTGTTTTTCTCCACAAAGTATCTAACAA 3700
3701 AACCAATGTGCAGACTGATTGGCCTGGTCATTGGTCTCCGAGAGAGGAGGTTTGCCTGTGATTTCCTAATTATCGCTAGGGCCAAGGTGGGATTTGTAAA 3800
3801 GCTTTACAATAATCATTCTGGATAGAGTCCTGGGAGGTCCTTGGCAGAACTCAGTTAAATCTTTGAAGAATATTTGTAGTTATCTTAGAAGATAGCATGG 3900
3901 GAGGTGAGGATTCCAAAAACATTTTATTTTTAAAATATCCTGTGTAACACTTGGCTCTTGGTACCTGTGGGTTAGCATCAAGTTCTCCCCAGGGTAGAAT 4000
4001 TCAATCAGAGCTCCAGTTTGCATTTGGATGTGTAAATTACAGTAATCCCATTTCCCAAACCTAAAATCTGTTTTTCTCATCAGACTCTGAGTAACTGGTT 4100
4101 GCTGTGTCATAACTTCATAGATGCAGGAGGCTCAGGTGATCTGTTTGAGGAGAGCACCCTAGGCAGCCTGCAGGGAATAACATACTGGCCGTTCTGACCT 4200
4201 GTTGCCAGCAGATACACAGGACATGGATGAAATTCCCGTTTCCTCTAGTTTCTTCCTGTAGTACTCCTCTTTTAGATCCTAAGTCTCTTACAAAAGCTTT 4300
4301 GAATACTGTGAAAATGTTTTACATTCCATTTCATTTGTGTTGTTTTTTTAACTGCATTTTACCAGATGTTTTGATGTTATCGCTTATGTTAATAGTAATT 4400
4401 CCCGTACGTGTTCATTTTATTTTCATGCTTTTTCAGCCATGTATCAATATTCACTTGACTAAAGTCACTCAATTAATCAATAAAAAAAAAAAAAAAAAA 4500
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Figure 8c

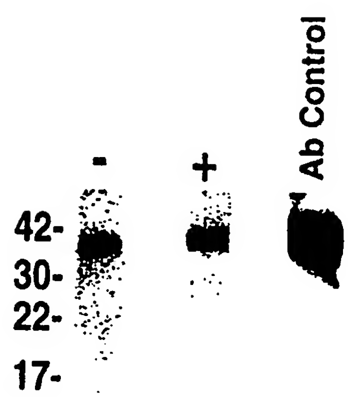

Figure 9

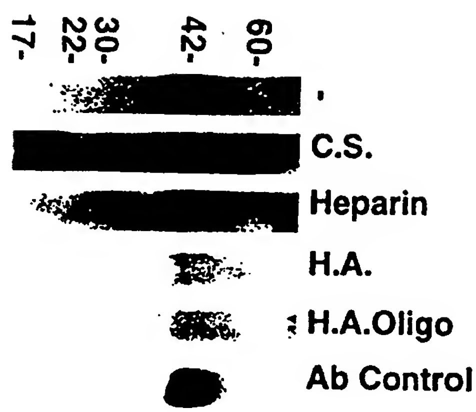

Figure 10

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GAATTCTCAGGAATTCGAGGTAGAAGGTGGCAGAGAGACTTCTGTTCCTGGGGGCCGAGCTGTTGTGCTGATACCGTCCTCTTGGCGTCTGCCCTAGTGG 100
GGACCCTTGATTTTAACTTGAAGTTCCTGGACTGGGTCTAACCTTAGCATGTGTGCCTGAGTGATGGACTTGGTATTTACACCAGCCACGCTGATAAGTG 200
CACATGTGTTTTTAATGTTTTGGCTTTCCACACCACAAACACACAGATGTGCTGTCGCCCGGGCTAGGACTTGAGTAGGGTTTTTCTATTTAAATATATA 300
TTATATATTTAAAAAAGTGTCCTCCCAGAGCTAATACCGTTGCTAGCAGCTCTTCCTGCCGCCACACCGGGCAAAGTCCACCCACTGCCCCAGTGTTGAG 400
GGCCACCATGGGCGGCCCCACCTGGAGAGGTGCTGCTCACAGCAAACAGCTCCAACTGCGCCTTCGCCTCGCCTTCCAGGGAGCCCAGCCAGGCCCACTG 500
GGTATTTACAAGCAGACCTCCCTCGCTTCAGCCTTCCTGAACCCCTGTTAGTTGGGAAACCACCTGTCTGCACCGCAGCTAGAGAACCGAGGAGAGGAGC 600
CGCTAGTCTAAAGGGCTGTTGGTTGAAATTAGGAAGCAGTGTAAAGAAAAAGAAAAAAAAAGTTTGGGAGGCCAAGGCAGGAGCATCACCTGAGGTCAGC 700
AGTTCGAGACCAGCCTGGCTAACGTGGTGAAACCCCGTCTCTACTAAAAATACAAAAAATTAGCGGGGGCGTGGTGGCACGCGCCTGTAATCCCAGCTAC 800
TCGGGAGGCTGAGGCAGGAGAATGGCTTGAACCCGGGAGGCGGAGGAAGCAGTCACGGAGATAGCGCCATTGCACTCCAGCTTAGGCAACAAGAGAGCGA 900
AACTTCGTCAAAAAAAAAAAGTCTTCATAATTTCATGGGTTTGCAAGTATGATCCAGGCTCCCCGCTTCTCTGCAAGCCAATGCGAGTTAATTACAGCGT1000
CCGCCCTGGTCTCTCTCCACCCCACGCCGTGATCCATTCCCCTTCTTTTTCTCCCCTTGTCTTTTCCTACTCCCCCTTTTATTTATGTATTTTTGGTTTT1100
GTTTTTTAAGGGGTGTTGAGCCGCGTCTGGTTCTAGTAAACCGAACCCGCTCGCGAGGGAGGCGATTGGCTCCCGCGCCGGTGACGGACGTGGTAACGAG1200
TGCGGCTCGCCCCGCCGGGAGCTGATTGGCTGCGCGGGGCGGCTCCGAGGGCTCGGCCGTAGGAGCCCCGCGCACTCCAGCCCTGCAGCCTCCGGAGTCA1300
GTGCCGCGCGCCCGCCGCCCCGCGCCTTCCTGCTCGCCGCACCTCCGGGAGCCGGGGCGCACCCAGCCCGCAGCGCCGCCTCCCCGCCCCGCGCCCGCCTCC1400
GACCGCAGGCCGAGGGCCGCCACTGGCCGGGGGACCGGGCAGCAGCTTGCGGCCGCGGAGCCGGGCAACGCTGGGGACTGCGCCTTTTGTCCCCGGAGG1500
TCCCTGGAAGTTTGCGGCAGGACGCGCGCGGGGAGGCGGCGGAGGCAGCCCCGACGTCGCGGAGAACAGGGCGCAGAGCCGGCATGGGCATCGGGCGCAC1600
                                                                                  FRP coding >>>>>>
GGAGGGGGGCCGCCGCGGGGCAGCCCTGGGCGTGCTGCTGGCGCTGGGCGGCGCTTCTGGCCGTGGGCTCGGCAGCGAGTACGACTACGTGAGCTTCCAG1700
TCGGACATCGGCCCGTACCAGAGCGGGCGCTTCTACACCAAGCCACCTCAGTGCGTGGACATCCCCGCGGACCTGCGGCTGTGCCACAACGTGGGCTACA1800
AGAAGATGGTGCTGCCCAACCTGCTGGAGCACGAGACCATGGCGGAGGTGAAGCAGCAGGCCAGCAGCTGGGTGCCCCTGCTCAACAAGAACTGCCACGC1900
CGGGACCCAGGTCTTCCTCTGCTCGCTCTTCGCGCCCGTCTGCCTGGACCGGCCCATCTACCCGTGTCGCTGGCTCTGCGAGGCCGTGCGCGACTCGTGC2000
GAGCCGGTCATGCAGTTCTTCGGCTTCTACTGGCCCGAGATGCTTAAGTGTGACAAGTTCCCGGAGGGGGACGTCTGCATCGCCATGACGCCGCCCAATG2100
CCACCGAAGCCTCCAAGCCCCAAG
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Figure 11

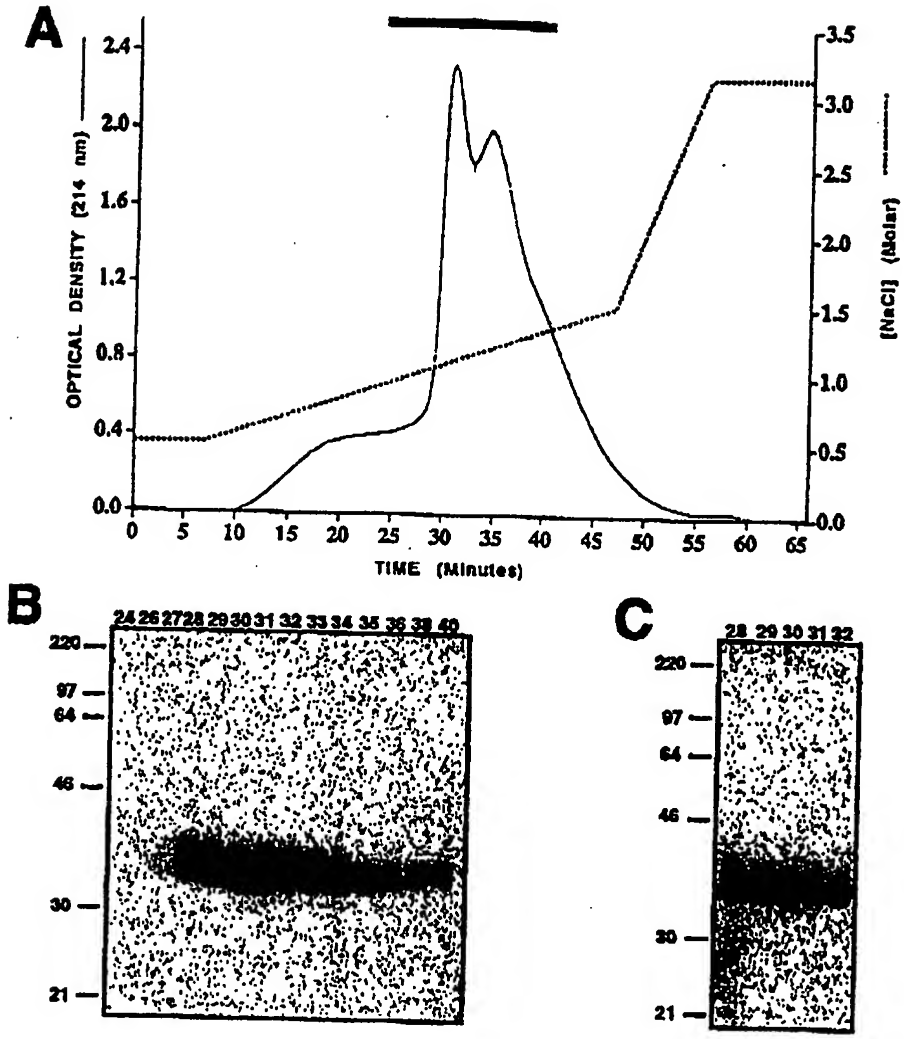
A
B
C
OPTICAL DENSITY (214 nm)
[NaCl] (Molar)
TIME (Minutes)
24 26 27 28 29 30 31 32 33 34 35 36 38 40
28 29 30 31 32
220
97
64
46
30
21

Figure 12

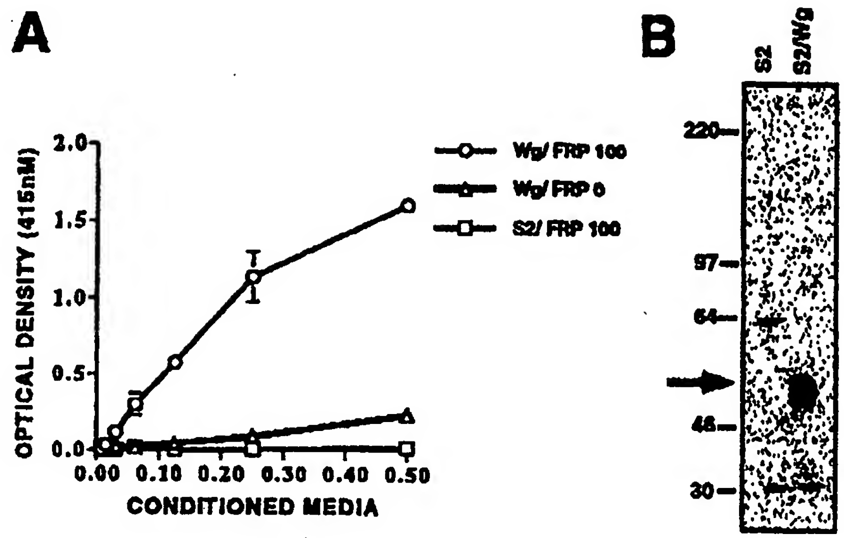
A
OPTICAL DENSITY (415nM)
2.0
1.5
1.0
0.5
0.0
0.00 0.10 0.20 0.30 0.40 0.50
CONDITIONED MEDIA
Wg/ FRP 100
Wg/ FRP 0
S2/ FRP 100
B
S2
S2/Wg
220
97
64
46
30

Figure 13

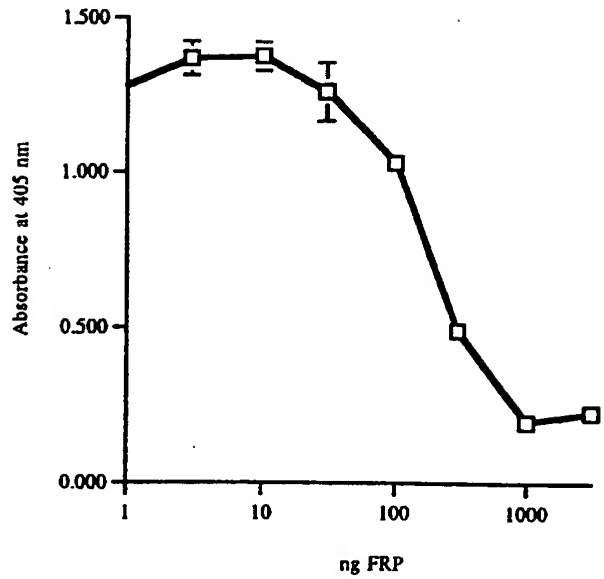

Figure 14

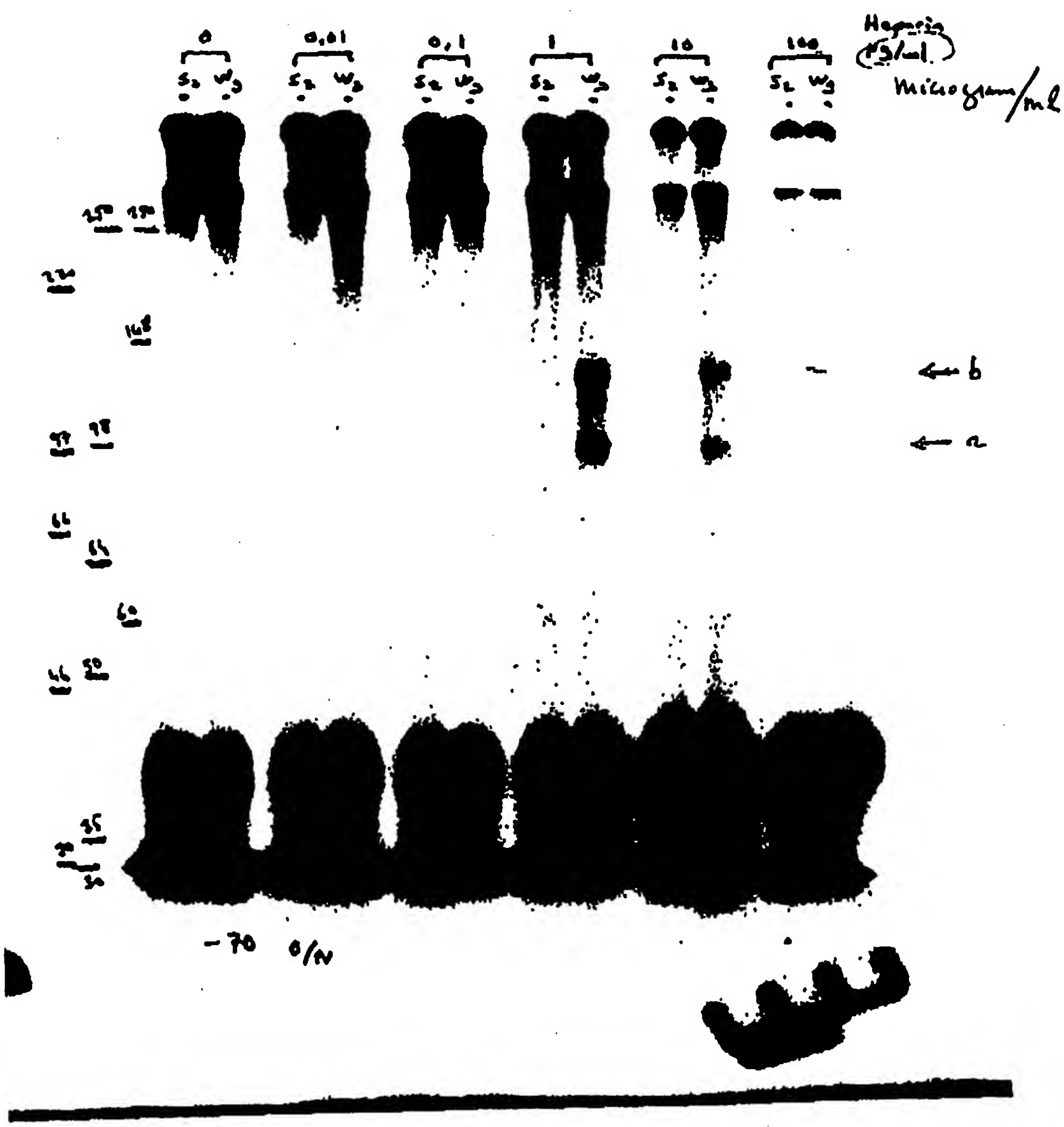

Figure 15

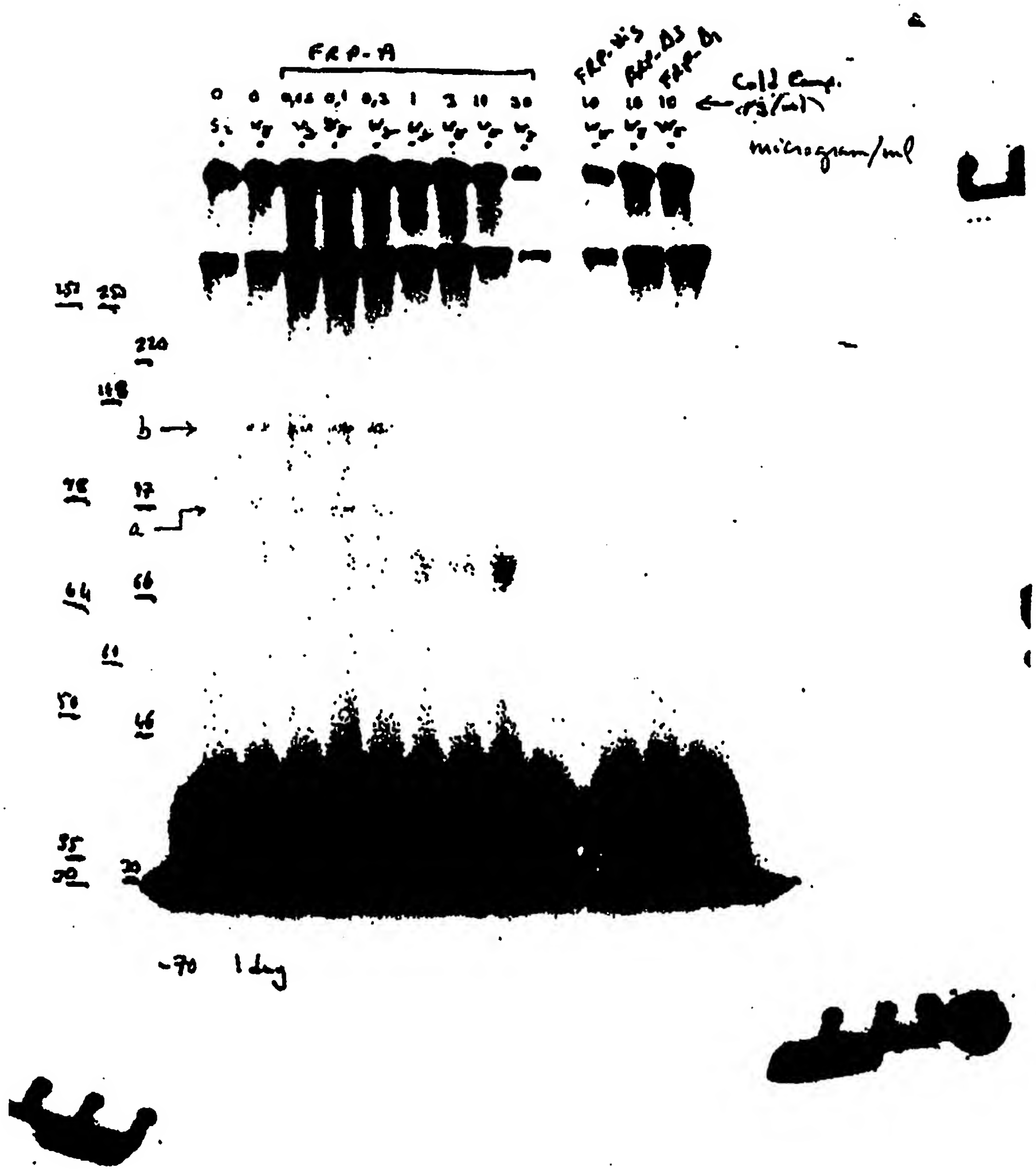

Figure 16

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/10974

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/11 C12N15/85 C12N5/10 C12Q1/68 C07K14/47 C07K16/18 C07K16/46 A01K67/027 G01N33/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N C12Q A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FUJIWARA T. ET AL.: "Otsuka cDNA project, AC C15983" EMBL DATABASE, 29 September 1996, XP002077643 Heidelberg see the whole document	17-20, 24-29
X	HILLIER L. ET AL.: "The WashU-Merck EST project, AC N75803" EMBL DATABASE, 5 April 1996, XP002077644 Heidelberg see the whole document	17-20, 24-29

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 1998	28/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HOANG B ET AL: "PRIMARY STRUCTURE AND TISSUE DISTRIBUTION OF FRZB, A NOVEL PROTEIN RELATED TO DROSOPHILA FRIZZLED, SUGGEST A ROLE IN SKELETAL MORPHOGENESIS" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 42, 18 October 1996, pages 26131-26137, XP002054777 cited in the application see the whole document ---	1-3,17, 18, 21-35, 37-39,45
X	RATTNER A ET AL: "A FAMILY OF SECRETED PROTEINS CONTAINS HOMOLOGY TO THE CYSTEINE-RICH LIGAND-BINDING DOMAIN OF FRIZZLED RECEPTORS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, no. 7, 1 April 1997, pages 2859-2863, XP002054779 cited in the application see the whole document ---	1-4, 11-18, 21-35, 37-39, 45-48
P,X	ZHOU Z. AND WANG J.: "AC AF056087" EMBL DATABASE,9 April 1998, XP002077645 Heidelberg see the whole document ---	1-5, 11-39
P,X	FINCH P W ET AL: "PURIFICATION AND MOLECULAR CLONING OF A SECRETED, FRIZZLED-RELATED ANTAGONIST OF WNT ACTION" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 94, 24 June 1997, pages 6770-6775, XP002057809 see the whole document ---	1-48
P,X	WO 98 13493 A (UMANSKY SAMUIL ;MELKONYAN HOVSEP (RU); LXR BIOTECHNOLOGY INC (US)) 2 April 1998 see the whole document -----	1-48

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 98/10974

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813493 A	02-04-1998	AU 4651397 A	17-04-1998